Hypertension

Catecholamines Regulate the Activity, Secretion, and Synthesis of Renalase

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Background—We previously identified renalase, a secreted novel amine oxidase that specifically degrades circulating catecholamines. Parenteral administration of either native or recombinant renalase lowers blood pressure, heart rate, and cardiac contractility by metabolizing circulating catecholamines. Renalase plasma levels are markedly reduced in patients with chronic kidney disease. It is not known whether endogenous renalase contributes to the regulation of catecholamines.

Methods and Results—We show here that circulating renalase lacks significant amine oxidase activity under basal conditions (prorenalase) but that a brief surge of epinephrine lasting <2 minutes causes renalase activity to increase from 48±18 to 2246±98 arbitrary units (n=3; P<0.002). Enzyme activation is detectable within 30 seconds and sustained for at least 60 minutes. Analysis of epinephrine-mediated hemodynamic changes in normotensive rats indicates that prorenalase becomes maximally activated when systolic pressure increases by >5 mm Hg. The catecholamine surge also leads to a 2.8-fold increase in plasma renalase concentration. Cultured cells exposed to dopamine upregulate steady-state renalase gene expression by >10-fold. The time course of prorenalase activation is abnormal in rats with chronic kidney disease.

Conclusions—These data identify a novel mechanism for the regulation of circulating catecholamines. In the renalase pathway, excess catecholamine facilitates the conversion of prorenalase, an inactive plasma amine oxidase, to renalase, which can degrade catecholamines. Excess catecholamines not only regulate the activation of prorenalase but also promote its secretion and synthesis. Because chronic kidney disease is associated with a number of systemic abnormalities, including activation of the sympathetic nervous system, increased catecholamines levels, cardiac hypertrophy, and hypertension, renalase replacement is an attractive therapeutic modality owing to its role in catecholamine metabolism. (Circulation. 2008;117:1277-1282.)

Key Words: catecholamines ■ hypertension ■ kidney diseases ■ physiology

Clinical studies indicate that patients with either stage 3 through 5 chronic kidney disease (CKD) or end-stage renal disease (ESRD) receiving renal replacement therapy are at significantly higher risk for developing cardiovascular disease.1–4 A number of causative conditions have been identified, including traditional risk factors such as hypertension and hyperlipidemia. Additional risk factors such as increased oxidative stress,5 heightened sympathetic tone,6,7 and arterial calcification also appear to play a key role in the development of cardiovascular disease in the CKD population.

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Patients with CKD stage 3 and above develop increased sympathetic tone, which can be documented by microneurography, a technique used to monitor sympathetic nerve activity.8–10 The increased sympathetic nerve activity can, at least in part, explain the increased in plasma catecholamines (catecholamine overspill) observed in CKD patients. It is also known that catecholamine clearance is decreased in CKD. For example, norepinephrine clearance is reduced by 20% in mild renal failure and by up to 40% in patients on hemodialysis.11

Our recent identification of renalase—a novel flavin adenine dinucleotide–dependent amine oxidase that is secreted into the blood by the kidney,12 metabolizes circulating catecholamines, and is deficient in CKD—provides a plausible theory for the increased catecholamines levels observed in CKD. Renalase resides on chromosome 10 at q23.33 and encodes a 342–amino acid protein with a calculated molecular mass of ~38 kDa. It is a distant relative of monooamine oxidase (MAO)-A with <14% amino acid identity. The renalase protein has been very well conserved throughout evolution, with orthologs not only in chimpanzee (95% amino acid identity) but also in Cyanobacteria (23% identity). It degrades catecholamines in vitro with a distinct substrate specificity and inhibitor profile, indicating that it represents a new class of flavin adenine dinucleotide–containing monoamine oxidases. Renalase can be detected in human...
plasma by Western blotting. It lowers blood pressure in vivo by decreasing cardiac contractility and heart rate and by preventing the expected compensatory increase in peripheral vascular tone. Because renalase blood levels are markedly reduced in ESRD patients receiving hemodialysis, the kidney appears to be the major source of circulating renalase. Renalase deficiency may contribute to the heightened sympathetic tone observed in ESRD patients.

In the present study, we assess the role of renalase in maintaining catecholamine homeostasis in vivo and show that blood renalase activity parallels that of the sympathetic nervous system. Therefore, under basal conditions when catecholamine levels are low, blood renalase activity is nearly undetectable. Conversely, increased catecholamine levels up-regulate the activity, secretion, and synthesis of renalase.

Methods

Antibody Preparation
An anti-renalase polyclonal antibody was generated with recombinant glutathione S-transferase-renalase fusion protein as the antigen (Proteintech Group Inc, Chicago, Ill). The antibody was purified by affinity chromatography with a column containing full-length renalase.

Western Blot Analysis
Studies were carried out as previously described with the anti-renalase antibody.

Immunolocalization
Protein expression in human kidney and heart was examined by use of the anti-renalase antibody as previously described.

Human Renalase Purification
Ammonium sulfate was added to a final concentration of 40% to 2 L urine collected from normal volunteers. After an overnight incubation at 4°C, the pellet was collected by centrifugation (10,000g for 30 minutes), resuspended in cold PBS buffer containing protease inhibitors, and dialyzed overnight at 4°C. Renalase was purified with an agarose–anti-renalase affinity column. The purified protein was eluted by use of an Immunopure IgG elution (Pierce Biotechnology, Woburn, Mass), analyzed by SDS-PAGE, and used in in vitro amine oxidase assays.

Amine Oxidase Assay
We assessed the ability of renalase to oxidize biogenic amines using an Amplex Red Monoamine Oxidase Assay Kit (Invitrogen, Carlsbad, Calif). The assay is based on the detection of H2O2 in a horseradish peroxidase–coupled reaction using 10 acetyl-3,7-dihydroxy-phenoxazine (Amplex Red reagent, Invitrogen). The Amplex Red reagent reacts with H2O2 in a 1:1 stoichiometry, and the resulting fluorescence signal is directly proportional to H2O2 production and hence amine oxidase enzymatic activity. Experiments were carried out according to the manufacturer’s instructions, with a final substrate concentration of 2 mmol/L. Amine oxidase activity that is specific to renalase was determined by use of a polyclonal antibody that inhibits renalase enzymatic function.

Catecholamine Infusion
Sprague-Dawley rats were obtained from a commercial vendor. Rats that had undergone removal of five sixths of the kidney tissue (5/6 Nx) were obtained from Charles River Laboratories (Wilmington, Mass). Rats (150 to 300 g) were anesthetized with Inactin (100 mg/kg; Sigma-Aldrich Co, St Louis, Mo). A catheter (PE-240) was placed in the trachea for airway protection and in the left jugular vein (PE-10) for intravenous infusion of a maintenance fluid solution consisting of normal saline with 2.25% bovine albumin at a rate of 1.5 mL per 100 g body weight per hour. Core temperature was monitored through a rectal thermometer, and body temperature was maintained at 37°C with a heating pad. Arterial pressure and pulse were continuously monitored through a PE-50 catheter inserted into the right carotid artery and connected to a pressure transducer (ADInstruments, Colorado Springs, Colo). Hemodynamic recordings were digitized, stored, and analyzed with a PowerLab/8SP data acquisition system (ADInstruments). The rats were allowed 1 hour to recover after completion of the surgical procedure, and the subsequent 30 minutes served as a control period.

The rats were divided into two groups: the control group received a 2-minute infusion of a saline solution containing a final concentration of epinephrine (Sigma-Aldrich Co, St Louis, Mo). The experimental group then received a 2-minute infusion of epinephrine, norepinephrine, or dopamine at a concentration designed to raise mean blood pressure by 15 to 20 mm Hg. The control groups were injected with either 0.5 mg BSA or 0.5 mg recombinant glutathione transferase in 0.5 mL PBS. Blood pressure and pulse were continuously measured and recorded. Blood samples were collected at various times from 30 seconds to 60 minutes. The animals were killed at the end of the infusion, and kidney and heart tissues were collected for Western blot analysis and immunocytochemistry.

A dose response for the effect of epinephrine on blood pressure and renalase activity was established by infusing epinephrine over a 2-minute period at doses ranging from 0.1 to 100 μg/kg into normotensive, anesthetized rats. Blood pressure and pulse were continuously monitored, and blood samples were obtained at baseline and 30 seconds and 5 minutes after the start of epinephrine infusion.

Statistical Analysis
Standard paired Student t tests were used for comparisons between 2 groups. Standard unpaired Student t tests were used for group comparisons at equivalent periods. When appropriate, nonparametric repeated-measures ANOVA (Friedman’s test) was used to evaluate statistical significance. When the Friedman’s test revealed statistical significance, Dunn’s test was used for pairwise comparisons. All data are mean±SEM, and values of P<0.05 were accepted as a statistically significant difference.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

The renalase gene is expressed in kidney, heart, skeletal muscle, and liver. The protein is secreted in blood and can be detected by Western blotting of plasma proteins separated by SDS-PAGE.

Under basal conditions, plasma renalase activity is undetectable (Figure 1). In contrast, under the same conditions, the same amount of urine renalase exhibits robust amine oxidase activity. Perhaps urinary renalase is processed differently than blood renalase, or alternatively, plasma could contain a substance that inhibits renalase activity. To test the latter possibility, mixing
studies were carried out, and as shown in Figure 1, the addition of plasma to urine significantly inhibited urinary renalase activity. In control studies, albumin alone had no effect on urinary renalase activity. These data indicate that renalase exists in plasma in a form henceforth called prorenalase that lacks enzymatic activity under basal conditions and may interact with an inhibitory substance in plasma.

Although the molecular identity of the putative inhibitor is currently unclear, these observations led us to hypothesize that in vivo blood renalase remains in an inactive state as prorenalase until stimulated by the appropriate signal(s). We tested the possibility that an increase in blood catecholamines represented such a signal. As shown in Figure 2A, basal renalase activity is undetectable in rat plasma. Within 1 minute of the initiation of either an epinephrine (Figure 2A) or a dopamine (Figure 2B) infusion (total infusion time of 2 minutes), renalase activity increased significantly and remained high long after the catecholamine infusion was terminated and blood pressure had returned to preinfusion levels. The increase in amine oxidase activity was due entirely to renalase activation because it was completely blocked by the anti-renalase antibody. These data indicate that acute increases in catecholamines levels cause a rapid and sustained increase in blood renalase activity.

Because the observed stimulation occurred in <1 minute, it is unlikely to be mediated by secretion of preformed renalase or through new protein synthesis. To determine whether catecholamines also regulated renalase secretion, blood levels were monitored by Western blot. Epinephrine infusion for 2 minutes caused a small decrease in renalase blood level at 1 minute and 5 minutes (Figure 2C). The increased level observed at 15 minutes and beyond is most likely due to renalase secretion by the kidney.

The effect of catecholamines on renalase gene expression was examined in a cell culture model. H9C2 cells maintained in culture were incubated with 20 μmol/L dopamine for up to 12 hours, and renalase gene expression was assessed by reverse-transcription polymerase chain reaction. As shown in Figure 2D, a 12-hour incubation with dopamine caused a 10-fold increase in renalase message. Taken together, these data indicate that catecholamines regulate renalase through at least 3 distinct mechanisms: They acutely (within 1 minute) stimulate enzymatic activity in blood, increase secretion of preformed renalase within 15 minutes, and activate gene transcription within 12 hours.

To assess whether prorenalase could be activated by changes in catecholamines levels expected to occur in normal physiology, a dose-response curve was carried using epinephrine doses ranging from 0.1 to 100 μg/kg, and changes in hemodynamic parameters were plotted against plasma renalse activity. Increases in systolic pressure correlated well with the catecholamine dose and ranged from 1 to 110 mm Hg above baseline. A 5-mm Hg increase in systolic pressure was associated with a rapid (30 second) and near-maximal activation of prorenalase (Figure 3A). Diastolic (Figure 3B) and mean (data not shown) pressures showed no significant correlation with renalase activity. These data indicate that epinephrine-mediated increases in systolic pressure that are well within the physiological range are associated with rapid and significant activation of prorenalase.

Previous studies have documented that rats subjected to removal of ~85% of kidney tissue (5/6 Nx) develop progressive renal failure and many of the associated abnormalities, including increased sympathetic activation, hypertension, and left ventricular hypertrophy. The 5/6 Nx rat is considered an excellent animal model of CKD. Therefore, we used this model to
examine the effect of CKD on blood renalase and its regulation by circulating catecholamines. The 5/6 Nx rats develop severe blood renalase deficiency 2 to 3 weeks after surgery (Figure 4A). Renalase levels in kidney and heart tissues were examined by immunocytochemistry and Western blot. As depicted in Figure 4B, tissue levels were significantly reduced. Interestingly, kidney renalase migrates predominantly as a dimer (70 kDa) under reducing conditions in SDS-PAGE gels, whereas heart renalase runs as a monomer (35 kDa) under the same conditions. The decrease in tissue renalase also was evident in immunocytochemical studies (Figure 4C). We then examined whether renalase regulation was abnormal in 5/6 Nx rats by comparing the effect of epinephrine infusion on prorenalase activation in normal and CKD rats. As shown in Figure 4D, although epinephrine infusion can activate blood prorenalase in 5/6 Nx rats, the magnitude and duration of the activation were significantly lower than observed in control rats. These data indicate that rats with CKD have diminished levels of tissue and circulating prorenalase. Furthermore, the magnitude and time course of prorenalase activation by catecholamines are markedly reduced in these animals. Taken together, these data demonstrate that there is a marked decrease in renalase protein and renalase activity in 5/6 Nx rats and that the renalase deficiency may contribute to elevated plasma catecholamine levels.

**Discussion**

This work identifies a novel pathway that contributes to the homeostatic control of circulating catecholamines. Blood renalase activity mirrors sympathetic tone, whereas brief increases in catecholamine level upregulate the activity, secretion, and synthesis of renalase, a soluble amine oxidase, which specifically degrades circulating catecholamines and has significant in vivo hemodynamic effects.

The molecular mechanisms that mediate the acute activation of prorenalase in vivo are unclear. One possibility involves the proteolytic cleavage of blood prorenalase. Because the flavin adenine dinucleotide binding site is at the amino terminus (amino acid 3 to 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 destroying enzymatic activity. Perhaps a small segment can be removed at either the amino or carboxyl terminus. Alternatively, increased catecholamines could cause a conformational change in the prorenalase molecule with the subsequent binding of a circulating activator or dissociation of an inhibitor. The mixing studies described in this study strongly support the notion that plasma contains a substance that inhibits renalase activity. The molecular identity of this substance is unclear, as is the nature of its interaction with prorenalase. Finally, enzymatic activity could be regulated...
through the formation of renalase homomultimers or heteromultimers such as dimers and larger complexes. The dimer hypothesis is attractive because the active forms of many amine oxidases, including MAO-A and MAO-B, are dimers. Under denaturing and reduced conditions, plasma renalase migrates as a 37-kDa band, which corresponds to the predicted size of a monomer. Renalase protein cannot be visualized in urine unless it is immunopurified and concentrated as described in Methods, indicating that the amount of renalase in urine is far below that present in plasma. Of note, the predominant species of urine renalase runs as a 70-kDa band under reducing conditions in SDS-PAGE gels, suggesting the presence of a covalently linked dimer.

Previous studies using graded infusions of epinephrine and norepinephrine indicate that in humans these catecholamines accelerate their own metabolic clearance. A 2-fold increase in plasma epinephrine caused an 80% increase in clearance. This effect was observed with epinephrine levels ranging from 90 to 1020 pg/mL. It was further determined that the increased catecholamine clearance could be prevented by β-blockade with the nonselective inhibitor propranolol but not with β-inhibition with the nonselective blocker phentolamine. Because circulating renalase is rapidly activated by catecholamine infusion, it likely contributes to the observed tolerance by increasing catecholamine degradation and clearance. Other possible mechanisms include increased clearance by the kidney; increased peripheral uptake with subsequent degradation by MAO-A, MAO-B, and catechol-O-methyl transferase; and receptor internalization.

Sympathetic overactivity has been well documented in patients with chronic renal failure. Comparison of dialysis patients who had undergone bilateral nephrectomies with those who still had their native kidneys indicates that the diseased kidneys are a major source of the activating stimuli. Although bilateral nephrectomy has been reported to improve the control of blood pressure in ESRD and to be associated with a notable decrease in circulating angiotensin, blood pressure remains significantly elevated, and there is no evidence that the procedure can normalize systemic pressure. In 1 study, a significant change in mean blood pressure was noted at 3 months but not at 1 month after nephrectomy. The patients remained very hypertensive, and in no patient did blood pressure normalize. Renalase levels are markedly reduced in patients with ESRD, whereas angiotensin levels can be significantly elevated. The extent to which bilateral nephrectomy would be expected to further reduce renalase levels is unclear because renalase is expressed in other tissues, which could contribute to the steady levels in ESRD. Although a further reduction in steady-state renalase level may occur in patients with ESRD who undergo bilateral nephrectomy, it would probably be outweighed by a concomitant and dramatic reduction in sympathetic output and in angiotensin levels. Experimental data point to significant cross-talk between the sympathetic nervous system and kidney. The sympathetic nervous system modulates renal hemodynamics via an α1-adrenergic receptor–mediated pathway, directly increases transstubular sodium reabsorption mediated through α2- and α1-adrenergic receptors located in the peritubular membranes, and stimulates renin release from the juxtaglomerular granular cells mediated via β1-adrenergic mechanisms. The kidney generates activating afferent signals by stimulating chemoreceptors and baroreceptors. These signals appear to gain strength and to last longer when the kidney is injured. Plasma dopamine and norepinephrine levels and sympathetic tone are consistently increased in patients with ESRD. Elevated sympathetic tone is believed to contribute to the pathogenesis of cardiovascular complications such as hypertension, left ventricular hypertrophy, and dysfunction.

Recent animal studies provide further evidence for the hypothesis that renalase plays a key role in the regulation of sympathetic tone and blood pressure. Renalase gene expression and protein expression were decreased by 70% and 40%, respectively, in rats treated with renalase antisense RNA. The decrease in renalase levels was associated with a 13-mm Hg increase in systolic blood pressure, and norepinephrine infusion had a significantly greater pressor effect in the renalase-deficient animals, increasing blood pressure by 64 mm Hg in these animals compared with a 33-mm Hg increase in controls. These data suggest that renalase plays a key role in the regulation of blood pressure by modulating the pressor response of the adrenergic system. Salt-sensitive Dahl rats develop hypertension when fed a high-salt diet. Blood and kidney tissue renalase levels were significantly decreased in salt-sensitive Dahl rats maintained on an 8% salt diet for 3 weeks. The salt-fed animals had a 3-fold increase in norepinephrine levels, became hypertensive, and developed proteinuria. Taken together, these data further support our hypothesis that renalase plays a role in regulating catecholamine metabolism and may contribute to the pathogenesis of hypertension.

We believe that identifying renalase is an important step toward a finer understanding of the kidney, of catecholamine metabolism, and of cardiovascular physiology, and we propose the renalase pathway depicted in Figure 5. It is well established that extracellular catecholamines are taken up by the cells and metabolized with MAO and catechol-O-methyl transferase. Our data indicate that they also stimulate the synthesis and secretion of renalase. Circulating prorenalase is inactive until it is acted on, in some fashion, by catecholamines. Once activated, renalase degrades extracellular cate-
Cholamines. Thus, the renalase pathway provides an additional means of regulating circulating catecholamine levels. Renalase deficiency in CKD suggests a causal link to the heightened sympathetic tone and increased cardiovascular risk that are well documented in this condition. We speculate that renalase replacement therapy will improve the cardiovascular outcome in CKD by providing an important mechanism for regulating the sympathetic nervous system.

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Disclosures
Dr Xu holds a patent on renalase submitted through Yale University. Drs Li and Wang may receive royalty income from a Yale patent. Dr Velazquez has received a research grant from Ren Pharmaceuticals, is listed as an inventor on a renalase patent through Yale University, has served as a consultant owns stock in Ren Pharmaceuticals, and is listed as an inventor on a patent. The other authors report no conflicts.

References

**CLINICAL PERSPECTIVE**

The present work describes the renalase system, a previously unrecognized mechanism for regulating catecholamines, cardiac function, and blood pressure. In this pathway, the kidney secretes prorenalase into the blood and is primarily responsible for maintaining steady levels of the enzyme. Although prorenalase has no detectable amine oxidase active in the basal state, it is rapidly converted to renalase by increased catecholamines and systolic blood pressure. Renalase in turn degrades catecholamines and decreases blood pressure. Excess catecholamines not only regulate the activation of prorenalase but also promote its secretion and synthesis. Not surprisingly, abnormalities in the renalase pathway occur in chronic kidney disease and end-stage renal disease, as evidenced by decreased plasma levels in humans and in rats subjected to subtotal nephrectomy. Unexpectedly, however, renalase deficiency was documented not only in kidney tissue but also in the heart of rats with chronic kidney disease. Assuming that a similar reduction in cardiac renalase occurs in patients with chronic kidney disease and end-stage renal disease, we speculate that it could account for the increased risk of ischemic and arrhythmic events described in this population. It also has been reported that renalase deficiency occurs early in the development of salt-dependent hypertension before significant renal damage has occurred. Collectively, these data suggest that renalase deficiency contributes importantly to the development and maintenance of hypertension and is associated with an increased risk for cardiovascular events. Therefore, renalase replacement therapy may modify cardiovascular risks.
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