

Renalase is a novel, soluble monoamine oxidase that regulates cardiac function and blood pressure

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The kidney not only regulates fluid and electrolyte balance but also functions as an endocrine organ. For instance, it is the major source of circulating erythropoietin and renin. Despite currently available therapies, there is a marked increase in cardiovascular morbidity and mortality among patients suffering from end-stage renal disease. We hypothesized that the current understanding of the endocrine function of the kidney was incomplete and that the organ might secrete additional proteins with important biological roles. Here we report the identification of a novel flavin adenine dinucleotide–dependent amine oxidase (renalase) that is secreted into the blood by the kidney and metabolizes catecholamines in vitro (renalase metabolizes dopamine most efficiently, followed by epinephrine, and then norepinephrine). In humans, *renalase* gene expression is highest in the kidney but is also detectable in the heart, skeletal muscle, and the small intestine. The plasma concentration of renalase is markedly reduced in patients with end-stage renal disease, as compared with healthy subjects. Renalase infusion in rats caused a decrease in cardiac contractility, heart rate, and blood pressure and prevented a compensatory increase in peripheral vascular tone. These results identify renalase as what we believe to be a novel amine oxidase that is secreted by the kidney, circulates in blood, and modulates cardiac function and systemic blood pressure.

Introduction

In addition to maintaining fluid and electrolyte homeostasis, the kidney also serves as an endocrine organ and is, for example, the main source of erythropoietin. Erythropoietin is required for proliferation and terminal differentiation of erythroid progenitors and precursors and is a major determinant of red cell mass (1, 2, 3). The kidney is the most important site for the release of renin, an enzyme that cleaves angiotensinogen to angiotensin I (4). The renin-angiotensin system is a key regulator of fluid and electrolyte metabolism, blood pressure, and cardiac function. An increase in sympathetic stimulation or a decrease in blood flow or in sodium chloride delivery to the distal tubules can stimulate the release of renin.

Patients who develop end-stage renal disease (ESRD) are either treated with replacement therapy, such as peritoneal or hemodialysis, or receive a renal transplant. Despite the success of dialysis in prolonging life, the morbidity and mortality associated with this therapy remain high, and most patients experience a poor quality of life (5, 6). While the reasons for this are not entirely clear, it is generally believed that the procedure fails to replicate important functions of the natural organ. For instance, it is well documented that patients with ESRD are at significantly higher risk for developing cardiovascular disease, a risk that appears to be correlated with increased oxidative stress (7) and heightened sympathetic tone (8, 9).

We hypothesized that the current understanding of the endocrine function of kidney was incomplete and that the organ might secrete additional proteins with important biological roles. Here we report the identification of a novel flavin adenine dinucleotide–dependent (FAD-dependent) amine oxidase (renalase) that is secreted into the blood by the kidney, metabolizes circulating catecholamines, and regulates blood pressure.

Results

Identification of renalase. In order to identify novel proteins secreted by the kidney, we analyzed all the clones published by the Mammalian Gene Collection Project (MGC) (10). As of August 1, 2003, there were 12,563 distinct genes derived from 77 different human cDNA libraries. We identified a total of 114 candidate genes encoding novel secretory proteins based on the following criteria: they encode proteins (a) with less than 20% sequence similarity/identity to known proteins; (b) that are predicted (according to SignalP-2.0 and SOSUIsignal Beta Version) to contain a signal peptide sequence; and (c) that do not contain transmembrane domains (since some membrane proteins, such as type I membrane proteins, also harbor a signal peptide sequence). We then performed Northern blot analysis to assess the tissue expression pattern for each gene and found 1 clone with robust and preferential expression in human kidney (MGC12474; GenBank accession number BC005364) (Figure 1A). The major band (1.5 kb) is visible in heart, skeletal muscle, kidney, and liver. Two additional weaker bands are also detected; 1 is approximately 2.4 kb and only presents in skeletal muscle. The other is approximately 1.2 kb and is present in kidney and liver. These mRNA species may represent alternative splice variants. MGC12474 has 1,474 nt, and

Nonstandard abbreviations used: ESRD, end-stage renal disease; FAD, flavin adenine dinucleotide; GST, glutathione synthase; MAO-A, monoamine oxidase A; MGC, Mammalian Gene Collection Project; TAP, transcriptionally active PCR.

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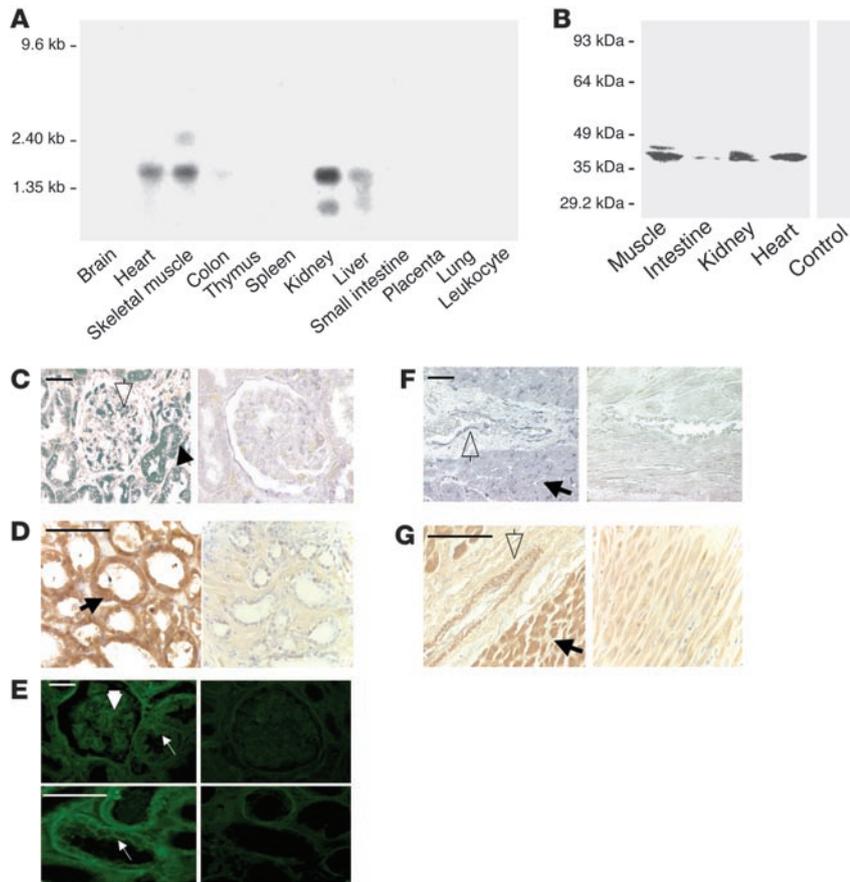


Figure 1
Tissue expression of renalase. **(A)** Northern blot analysis of human tissues using the MGC12474 clone as a probe. The upper band in skeletal muscle and lower bands in kidney and liver may represent alternatively spliced forms of renalase. **(B)** Western blot analysis of rat tissues using a renalase polyclonal antibody. **(C–F)** Renalase expression in kidney. **(C)** In situ hybridization analysis of human kidney. Left: antisense probe. Open arrow indicates the glomerulus; filled arrow indicates proximal tubules. Scale bar: 40 μ m. Right: sense probe control. Magnification, $\times 200$. **(D)** Immunolocalization in human kidney. Left: anti-renalase antibody. Filled arrow indicates proximal tubules. Scale bar: 40 μ m. Right: preimmune serum. Magnification, $\times 630$. **(E)** Immunofluorescence in human kidney. Left panels: anti-renalase antibody, thick arrow denotes the glomerulus; thin arrows indicate proximal tubules. Right panels: preimmune serum. Scale bars: 40 μ m. **(F and G)** Renalase expression in heart. **(F)** In situ hybridization analysis of human heart. Left: antisense probe. Open arrow indicates blood vessels; filled arrow indicates ventricular myocytes. Scale bar: 40 μ m. Right: sense probe control. Magnification, $\times 200$. **(G)** Immunolocalization in human heart. Left: anti-renalase antibody. Open arrow indicates blood vessels; filled arrows indicate ventricular myocytes. Scale bar: 40 μ m. Right: preimmune serum. Magnification, $\times 630$.

its longest open reading frame (nt 22–1047) encodes a novel protein with 342 AAs with a calculated molecular mass of 37.8 kDa (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI200524066DS1). The cDNA sequence of MGC12474 was used to search the Human Genome Project database (<http://www.ncbi.nlm.nih.gov/genome/guide/human/>), and the resulting alignment was used to establish the exon-intron structure of the human gene, which we have named renalase. Renalase has 9 exons spanning approximately 311,000 bp and resides on chromosome 10 at q23.33. We examined the expression of renalase protein in rats by Western blotting using an anti-renalase antibody. As shown in Figure 1B, renalase pro-

tein was detected in the same tissues that were determined to be positive for renalase mRNA by Northern blotting. In situ hybridization and immunocytochemical studies were carried out to determine the spatial distribution of renalase gene expression in human tissues. A specific signal was detected in renal glomeruli, proximal tubules (Figure 1, C–E), and cardiomyocytes (Figure 1, F and G).

Analysis, using MotifScan (<http://hcv.lanl.gov/content/hcv-db/MOTIFSCAN/MotifScanner.html>), revealed a signal peptide at the N-terminus, a FAD-binding site (AAs 4–35), and an amine oxidase domain at AAs 75–339 (Supplemental Figure 1). Renalase has 13.2% AA identity with monoamine oxidase A (MAO-A) (Supplemental Figure 2).

Renalase is a secreted protein. To test whether renalase is a secreted protein, we employed a PCR-based approach to generate transcriptionally active PCR (TAP) fragments (11). In order to facilitate the detection of the protein product, we also engineered an HA tag at the C-terminus of renalase. HEK293 cells that had been transfected with a renalase TAP fragment were capable of secreting renalase in the culture medium. As shown in Figure 2A, Western blotting with both anti-HA and anti-renalase antibodies revealed an approximately 35-kDa protein in the culture medium, which indicates that renalase has a functional N-terminal signal sequence and is secreted in the cell culture model used in these studies. We reasoned that if renalase is secreted in vivo, it should be detectable in either urine or blood. Human plasma was examined by Western blotting using a renalase-specific polyclonal antibody. As shown in Figure 2B, renalase was readily detectable in plasma of healthy individuals. To determine whether the kidney is an important source of secreted renalase, we examined the blood levels of renalase in patients suffering from severe kidney disease and decreased renal function. As shown in Figure 2B, renalase was virtually undetectable in the blood of patients with ESRD on hemodialysis.

Renalase degrades catecholamines in vitro and regulates systemic blood pressure in vivo. Structural anal-

ysis revealed that renalase contains an amino oxidase domain (Supplemental Figure 1), which suggests that it may play a role in amine oxidation. Therefore, we tested whether it had oxidase activity using a battery of amines as substrates. As shown in Figure 2C, renalase specifically metabolizes catecholamines, with dopamine being the preferred substrate, followed by epinephrine, and then norepinephrine. Its enzymatic activity was unaffected by known inhibitors of the FAD-containing amine oxidases MAO-A and MAO-B (Figure 2D). In control studies, pargyline and clorgyline inhibited MAO-A activity by $83.9\% \pm 2.3\%$ ($n = 3$) and $82.4\% \pm 1.9\%$ ($n = 3$), respectively.

To examine the oxidase activity of native renalase, and to verify the specificity of the anti-renalase antibody, we purified renalase

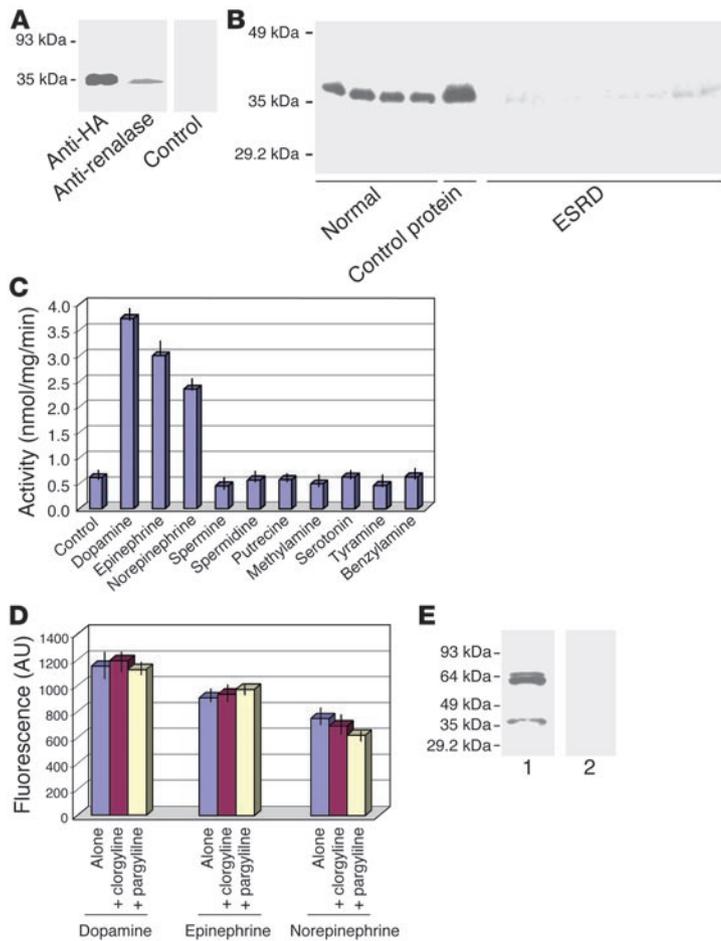


Figure 2

Renalase is a secreted amine oxidase. **(A)** Detection of renalase in culture medium of HEK293 cells transiently transfected with renalase cDNA. Control, secondary antibody alone. **(B)** Western blot analysis of human plasma using an anti-renalase antibody. Normal, plasma from individuals with normal renal function; Control protein, human recombinant renalase protein; ESRD, plasma from patients with ESRD receiving hemodialysis. **(C)** Renalase metabolizes catecholamines. Ten micrograms of GST-renalase fusion protein was used for each assay; amine oxidase is expressed as H₂O₂ production (nmol/mg/min). **(D)** Renalase activity is insensitive to known monoamine oxidase inhibitors. The renalase activity was assessed as described in **C**, with dopamine, epinephrine, and norepinephrine as substrates. The renalase activity was examined in the absence of inhibitor (Alone), in the presence of 1 μM clorgyline, or in the presence of 1 μM pargyline. Renalase activity is expressed as arbitrary fluorescence units/10 μg protein. In control studies, clorgyline (1 μM) and pargyline (1 μM) inhibited MAO-A activity by 83.9% ± 2.3% (n = 3) and 82.4% ± 1.9% (n = 3), respectively. **(E)** Affinity purification of human renalase. The anti-renalase polyclonal antibody was used to isolate protein from human urine. Lane 1: renalase from human urine; lane 2: control with secondary antibody alone.

from human urine and studied its activity. As shown in Figure 2E, in addition to the band of the expected size (approximately 35 kDa), another, larger (67–75 kDa) doublet was also detected, and it may represent either dimerization or aggregation of the 35-kDa band. We then used a functional assay to confirm the identity of the bands detected by the polyclonal antibody. The immunoprecipitate metabolized catecholamines with a substrate specificity similar to that of recombinant renalase (with dopamine being the preferred substrate, followed by epinephrine, and then norepinephrine; n = 4). Furthermore, we found that the anti-renalase antibody inhibited the amino oxidase activity of recombinant renalase by 87.3% ± 2.3% (n = 4). Since renalase circulates in blood and degrades catecholamines in vitro, we examined its in vivo effect on cardiovascular hemodynamics. As shown in Figure 3A and Table 1, within 30 seconds of a single bolus injection of recombinant renalase, systolic, diastolic, and mean arterial pressure decreased by 23.5% ± 1.3%, 32.6% ± 2.9%, and 28.9% ± 2.7% respectively (n = 8; P < 0.001). Blood pressure recovered to baseline values within 4 ± 1 minutes (n = 8). To further study the mechanism of renalase-mediated hypotensive effect, we monitored a number of hemodynamic parameters. As shown in Figure 3, A and B, and detailed in Table 1, renalase decreased left-ventricular end systolic and end diastolic pressure, maximum left ventricular pressure, and the rate of ventricular pressure change (dp/dt), a measure of cardiac contractility. In addition, renalase decreased heart rate, but peripheral vascular resistance remained unchanged. Renalase's

action on heart contractility and blood pressure was dose dependent (Figure 3, C and D).

Discussion

Renalase is a novel FAD-containing amine oxidase. It is critically dependent on FAD for oxidase activity, since the protein is inactive unless the cofactor is incorporated during protein production (0.1 μM FAD was added to the medium during the production of recombinant renalase). It has weak AA similarities to MAO-A and MAO-B and distinct substrate specificity and inhibitor profile, which indicates that it represents a new class of FAD-containing monoamine oxidases.

Amine oxidases are enzymes that metabolize biogenic amines and are classified according to the nature of the attached cofactor, such as FAD or topaquinone (TPQ). MAO-A and MAO-B are FAD-containing, mitochondrial enzymes that metabolize intracellular catecholamines. The crystal structure of human MAO-B has been determined at a resolution of 3.0 Å and reveals a dimer with the FAD cofactor covalently bound to a cysteine side chain (Cys-397) (12). MAO-A and MAO-B have overlapping substrate specificity; catabolize neurotransmitters such as epinephrine, norepinephrine, serotonin, and dopamine; and are specifically inhibited by clorgyline and deprenyl, respectively. Polyamine oxidase, the other known FAD-containing oxidase, is an intracellular oxidase that metabolizes spermine and spermidine and regulates cell growth (13). Unlike MAO-A and MAO-B, which are anchored through the carboxyl ter-

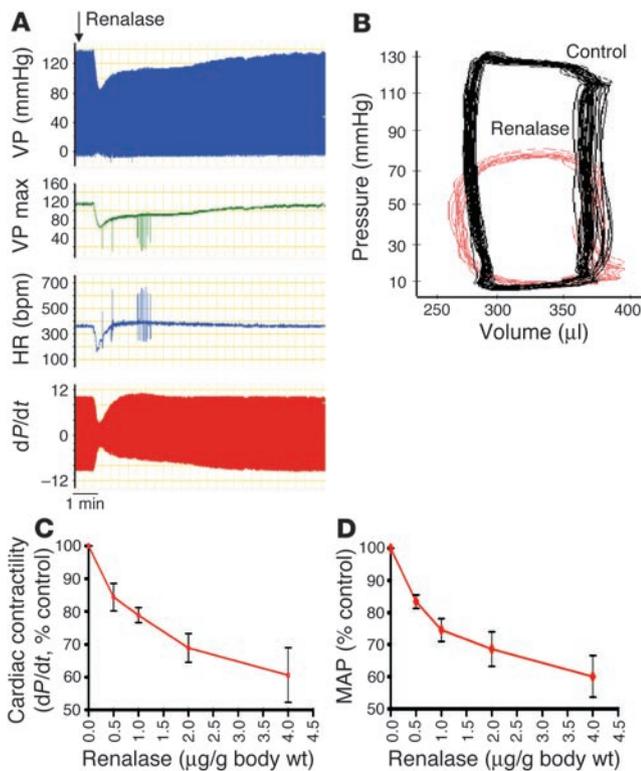


Figure 3

Effect on renalase of hemodynamic parameters. (A) Cardiac response before and after an i.v. bolus injection of 4 µg/g body wt. The arrow denotes the timing of renalase injection. VP, left ventricular pressure; VP max, maximal left ventricular pressure; HR, heart rate; dP/dt, rate of change in left ventricular pressure, a measure of cardiac contractility. (B) Pressure-volume changes before and after renalase injection. (C) Renalase dose-response curve: cardiac contractility. (D) Renalase dose-response curve: mean arterial pressure (MAP).

Native renalase purified from human urine is functionally active and retains enzymatic activity. Recombinant renalase lowers blood pressure when administered i.v. It is known that catecholamines control heart rate, myocardial contractility, and the tone of resistance vessels and thus play an important role in maintaining blood pressure. The hemodynamic changes observed with renalase infusion are most likely mediated by circulating catecholamines degradation, which would be expected to decrease cardiac contractility and heart rate. It is also possible that a significant fall in circulating catecholamines levels resulted in venous dilatation and decreased venous return. End diastolic pressure tended to be lower but the difference compared with control did not reach statistical significance. While the hypotensive effect of renalase can be fully accounted for by the observed decrease in contractility and heart rate, we cannot categorically exclude the possibility that renalase's effect may be partly receptor mediated.

Renal disease is associated with a marked increase in cardiovascular disease (16, 17). A number of hypotheses have been put forth to explain the increased frequency of cardiovascular dysfunction observed in patients with renal disease (18). Interestingly, some studies have shown that plasma dopamine and norepinephrine levels are consistently increased in patients with ESRD (9, 19–21). Those changes may contribute to the pathogenesis of cardiovascular complications such as hypertension, left ventricular hypertrophy, and dysfunction, which are important contributors to the high mortality rate observed in patients with ESRD. Thus, it is intriguing to speculate that low plasma renalase levels may also contribute to the heightened circulating catecholamine levels observed in ESRD patients.

In conclusion, our data indicate that renalase is a novel FAD-dependent amine oxidase that is secreted into the blood by the kidney. It degrades catecholamines in vitro and lowers blood pressure in vivo by decreasing cardiac contractility and heart rate and preventing a compensatory increase in peripheral vascular tone. Furthermore, the decrease in blood renalase concen-

minus to the outer mitochondrial membrane (14) and confined to intracellular compartments, renalase is secreted into the blood, where it is detectable by Western blotting.

Amine oxidase activity has been measured in human plasma and is believed to be mediated by vascular adhesion protein 1 (VAP-1), a copper-containing semicarbazide-sensitive amine oxidase that is secreted by smooth muscle cells, adipocytes, and endothelial cells (15). VAP-1's substrate specificity and inhibitor profile are very different from that of renalase. It metabolizes benzylamine and methylamine and is inhibited by semicarbazide and hydroxylamine. Therefore, renalase is the only known amine oxidase that is secreted into blood and that metabolizes circulating catecholamines.

The kidney appears to be the major source of circulating renalase, since the protein is nearly absent in the blood of patients with ESRD. This was an unexpected finding, as renalase gene and protein expression are also detectable, albeit at lower levels, in heart, skeletal muscle, and small intestine. Since the molecular details underlying the regulation of renalase secretion are unknown, one cannot exclude the possibility that the low circulating renalase level in ESRD patients is due to increased catabolism or to a generalized decrease in secretion brought about by the metabolic abnormalities associated with severe renal failure, i.e. the uremic state. Nonetheless, it is likely that the kidney is an important contributor to circulating renalase pool. It is interesting to note that skeletal muscle expresses high renalase levels, which suggests that the enzyme may play an important role in regulating local and perhaps systemic catecholamine concentration.

Table 1

Effect of renalase on hemodynamic parameters

	Control	Renalase	n	P
Mean arterial Pressure (mmHg)	106.4 ± 4.7	65.3 ± 3.5	8	<0.0001
End systolic pressure (mmHg)	127.7 ± 8.1	92.7 ± 2.7	5	<0.004
End diastolic pressure (mmHg)	11.3 ± 1.8	9.3 ± 1.5	5	NS
Heart rate (beats/min)	342 ± 6	304 ± 9	5	<0.004
Cardiac output (ml/min)	45.8 ± 2.8	33.2 ± 1.5	3	<0.03
dP/dt (mmHg/s)	8,604 ± 728	5,235 ± 442	5	<0.001
Arterial elastance (mmHg/µl)	0.94 ± 0.09	0.8 ± 0.04	3	NS
Systemic vascular resistance (mmHg/l/min)	2,323 ± 196	1,966 ± 183	3	NS

dP/dt, rate of change in left ventricular pressure, a measure of cardiac contractility.



tration observed in patients with severe renal disease suggests a causal link to the increased plasma catecholamine and heightened cardiovascular risk that are well documented in this patient population. The identification of renalase is an important step in developing a more detailed understanding of cardiovascular physiology and perhaps also in the quest for providing optimal treatment for patients with kidney disease.

Methods

Identification and analysis of the gene encoding renalase. As of August 1, 2003, 12,563 distinct human full-length open reading frame cDNAs were available from MGC's website. These clones were subjected to 3 rounds of sequential screening. First, genes without a GenBank definition were chosen for further analysis. Homology analysis was carried out using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST/), and genes having predicted amino acid sequences with less than 20% identity to known proteins were chosen for the next round of analysis. We identified approximately 2,500 clones after the first round of selection. Second, these 2,500 clones were examined for the presence of putative signal sequences using SignalP-2.0 (www.cbs.dtu.dk/services/SignalP-2.0/) and SOSUIsignal Beta Version (http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?/sosuisignal/sosuisignal_submit.html). We identified approximately 140 clones with putative signal peptides. Third, these 140 clones were further evaluated for the presence of transmembrane domains using SOSUIsignal Beta Version. Those containing transmembrane domains were excluded for further study. Thus, 114 clones remained and were then subjected to domain search using Protein Families Database of Alignments (Pfam) (<http://www.sanger.ac.uk/Software/Pfam/>). The cDNA clones of interest were purchased from the American Type Culture Collection (ATCC), sequenced on both strands (Yale University, Keck Foundation Biotechnology Resource Laboratory), and analyzed using BLAST.

Northern blot analysis. Studies were carried out with the entire coding region of the clones of interest as previously described (22).

Construction of gene expression cassettes. We used TAP to engineer a 5'-cytomegalovirus promoter and 3'-simian virus 40 polyadenylation signal site in each candidate clone (11). The following PCR primers were used to generate renalase TAP fragments: 5'-oligo, 5'-CTGCAGGCACCGTCGTC-GACTTAAACAatgacccagggcccccgcgcg (upper case represents the 5'-TAP universal sequence, which is used as an anchor for second-step PCR; lower case represents the renalase-specific sequence starting at the ATG initiation site); 3'-oligo, 5'-CATCAATGTATCTTATCATGTCTGATCAACCAGC-TACCATACGATGTTCCAGATTACGCTttttgtagttcttcaataag (the first 25 bases comprise 3'-TAP universal sequence, which is used as an anchor for second-step PCR; the underlined sequence encodes HA followed by a stop codon, TGA; lower case represents the renalase-specific sequence starting at 3' end minus the stop codon). The 5' and 3' primers used for the second-step PCR were provided by the manufacturer (Gene Therapy Systems Inc.).

Gene delivery and expression. In vitro transfection was carried out using GenePORTER (Gene Therapy Systems Inc.) following the procedures recommended by the manufacturer. We consistently obtained 40–60% transfection efficiency as assessed using a GFP TAP fragment as control.

In situ hybridization. Studies were performed as described previously (23). In brief, a 426-bp HindIII and PstI renalase fragment was cloned into the pBluescript II KS(+) vector. The vector was used to synthesize digoxigenin (DIG)-labeled RNA probes with DIG-labeling kit (Roche Biochemicals). The antisense was used to detect renalase gene expression, while the sense strand was used as control.

Generation of glutathione synthase–renalase recombinant protein. The renalase coding region (nt 22–1047) was amplified by PCR (sense primer, 5'-TTTT-GGATCCATGGCGCAGGTGCTGATCGTG and antisense, 3'-TTTT-

GAATTCCTAAATATAATTCTTTAAAGC). The PCR product was digested with BamHI and EcoRI and cloned into the pGEX-4T vector (Promega) in frame with a glutathione synthase (GST) tag (26 kDa) at the N-terminus. The clones were verified by DNA sequencing and used to transform *E. coli* BL21. Transformed bacteria were grown at 37°C for 16 hours in the presence of 0.1 μM FAD. Isopropyl-BD-thiogalactopyranoside (0.5 mM) was added for the last 3.5 hours of culture. The renalase fusion protein was purified using Glutathione Sepharose (Amersham Biosciences). When FAD was omitted during protein synthesis, the resulting synthetic renalase was devoid of oxidase activity (data not shown).

Antibody preparation. Anti-renalase polyclonal sera were generated by Pro-teinTech Group Inc., with recombinant GST-renalase fusion protein used as the antigen. The sera were purified by affinity chromatography using a column containing full-length renalase.

Western blot analysis. Studies were carried out as previously described using either anti-HA or anti-renalase antibodies (23).

Immunolocalization. Protein expression in human kidney and heart was examined using the anti-renalase polyclonal sera as previously described (23).

Amine oxidase assay. We assessed the ability of renalase to oxidize biogenic amines using an Amplex Red Monoamine Oxidase Assay Kit (Invitrogen Corp.). The assay is based on the detection of H₂O₂ in a HRP-coupled reaction using 10-acetyl-3,7-dihydroxy-phenoxazine (Amplex Red reagent; Invitrogen Corp.). 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 mM.

Human subjects. Plasma samples were obtained from 4 healthy individuals and 8 patients with ESRD. Among the ESRD patients, renal failure was due to chronic diabetic nephropathy ($n = 3$), hypertensive nephrosclerosis ($n = 3$), interstitial nephritis ($n = 1$), and autosomal dominant polycystic kidney disease ($n = 1$). Patients had been receiving hemodialysis treatment 3 times a week for at least 1 year. Blood samples were obtained prior to a routine dialysis treatment. None of the patients were receiving α or β blockers, clonidine, methyldopa, or MAO-A or MAO-B inhibitors, and none had a history of psychiatric disorders. The protocol was approved by the Human Investigative Committee of the Veteran Administration Connecticut Health Care System, and all participants gave written, informed consent.

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

Hemodynamics measurements. Sprague-Dawley rats (150–250 g each) were anesthetized with inactin (100 mg/kg). A polyethylene catheter (PE-240) was placed in the trachea for airway protection and in the left jugular vein (PE-50) for i.v. infusion of a maintenance fluid solution consisting of normal saline with 6.25% BSA at a rate of 1.5 ml/100 g body wt per hour. Core temperature was monitored through a rectal thermometer, and a heating pad was used to maintain body temperature at 37°C. Arterial pressure and pulse were continuously monitored through a PE-50 catheter inserted in the left carotid artery and connected to a pressure transducer (ADInstruments). Hemodynamic recordings were digitized, stored, and analyzed using a PowerLab/8SP data acquisition system (ADInstruments). The rats were allowed 1 hour to recover after the surgical procedure was completed, and the subsequent 30 minutes served as a control period. The experimental group then received a bolus injection of 0.5 mg of recombi-



nant renalase in 0.5 ml PBS. The control group was injected with either 0.5 mg BSA or 0.5 mg recombinant glutathione transferase in 0.5 ml of PBS. Blood pressure and pulse were continuously measured and recorded. To study renalase effect on cardiac function, we inserted a pressure/volume (P-V) combination catheter (Millar Mikro-Tip P-V catheter, model SPR-838; Millar Instruments) into the right common carotid artery and advanced it into the left ventricle in order to measure intraventricular pressures and volumes during the cardiac cycle. P-V loops were monitored continuously before and during infusion of renalase and recorded using a PowerLab data acquisition system (model 8SP; ADInstruments). Ventricular pressure and ventricular volume were measured, and heart rate, mean arterial pressure, cardiac output, dP/dt (cardiac contractility), arterial elastance, and systemic vascular resistance were calculated.

Statistical analysis. Standard paired Student's *t* tests were used for comparisons between two groups. Standard unpaired Student's *t* tests were used for group comparisons at equivalent periods. All data are mean values \pm SE, and $P < 0.05$ was accepted as a statistically significant difference.

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Supplementary Figures

Fig 1



Fig 2



SUPPLEMENTARY FIGURE LEGENDS

Figure 1

Nucleotide and deduced amino acid sequence (triple letter code) of human renalase and comparison with MAO-A.

Figure 2

Putative structural motifs detected in renalase: FAD: flavin adenine dinucleotide, SP: signal peptide.