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 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.

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 SOSUI signal 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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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. HER293 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 analysis 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% ± 2.3% (n = 3) and 82.4% ± 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 protein in rats by Western blotting using an anti-renalase antibody. As shown in Figure 1B, renalase expression of renalase protein in rats by Western blotting using anti-renalase antibody. Filled arrow indicates proximal tubules. Scale bar: 40 μm. Right: sense probe control. Magnification, ×200. (D) Immunolocalization in human kidney. Left: anti-renalase antibody. Open arrow indicates blood vessels; filled arrow indicates ventricular myocytes. Scale bar: 40 μm. Right: sense probe control. Magnification, ×200. (G) Immunolocalization in human heart. Left: anti-renalase antibody. Open arrow indicates blood vessels; filled arrows indicate ventricular myocytes. Scale bar: 40 μm. Right: preimmune serum. Magnification, ×630.

Figure 1

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). 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 protein 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/Motif-Scanner.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).

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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 83.9% ± 2.3% (\( n = 4 \)) 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.

**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 \( \mu \)M 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-
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 renal disease (18). 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 concent-

### Table 1

Effect of renalase on hemodynamic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Renalase</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial Pressure (mmHg)</td>
<td>106.4 ± 4.7</td>
<td>65.3 ± 3.5</td>
<td>8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>End systolic pressure (mmHg)</td>
<td>127.7 ± 8.1</td>
<td>92.7 ± 2.7</td>
<td>5</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>End diastolic pressure (mmHg)</td>
<td>11.3 ± 1.8</td>
<td>9.3 ± 1.5</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>342 ± 6</td>
<td>304 ± 9</td>
<td>5</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>Cardiac output (ml/min)</td>
<td>45.8 ± 2.8</td>
<td>33.2 ± 1.5</td>
<td>3</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>dP/dt (mmHg/s)</td>
<td>8,604 ± 728</td>
<td>5,235 ± 442</td>
<td>5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Arterial elastance (mmHg/μl)</td>
<td>0.94 ± 0.09</td>
<td>0.8 ± 0.04</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Systemic vascular resistance (mmHg/l/min)</td>
<td>2.323 ± 196</td>
<td>1.866 ± 183</td>
<td>3</td>
<td>NS</td>
</tr>
</tbody>
</table>

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 SOSUI signal Beta Version (http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?f=signalbeta?submit). We identified approximately 140 clones with putative signal peptides. Third, these 140 clones were further evaluated for the presence of transmembrane domains using SOSUI signal 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′-cyto-megalovirus promoter and 3′–simian virus 40 polyadenylation signal sequence in each candidate clone (11). The following PCR primers were used to generate renalase TAP fragments: 5′-oligo, 5′-CTGCAGGCCACCGTCGTCGACCTAAAACTGGGGAGCGGGGGCCG (upper case represents the 5′-unique gene 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′-CATCAATGTATCTTATCATGTCTGATCAACCAGCATTAAAACaaggctccccgggcccggcgg (upper case represents the 5′-unique gene 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′-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 Pstl 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′-TTTTGGATCCCATGGCCGACGTGCTGATCGTG and antisense, 3′-TTTTGGATCCTAAATATAATTTCTTTAAAGC). 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-B-D-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 Proteintech 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).

Immuno localization. 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 1-acetyl-3,7-dihydroxy-phenoxazone (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 renalse 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 ± SE, and P < 0.05 was accepted as a statistically significant difference.

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Jianchao Xu and Guoyong Li contributed equally to this work.

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.