Renalase deficiency aggravates ischemic myocardial damage

Yanling Wu[1,2,5], Jianchao Xu[1,2,5], Heino Velazquez[1,2], Peili Wang[1,2], Guoyong Li[1,2], Dinggang Liu[1], Benedita Sampaio-Maia[3], Janete Quelhas-Santos[3], Kerry Russell[1], Raymond Russell[1], Richard A. Flavell[4], Manuel Pestana[3], Frank Giordano[1] and Gary V. Desir[1,2]

[1]Department of Medicine, Yale University School of Medicine, New Haven, Connecticut, USA; [2]VACHS Medical Center, West Haven, Connecticut, USA; [3]Nephrology Research and Development Unit, Faculty of Medicine, Hospital S. João, Porto, Portugal and [4]Department of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut, USA

Chronic kidney disease (CKD) leads to an 18-fold increase in cardiovascular complications not fully explained by traditional risk factors. Levels of renalase, a recently discovered oxidase that metabolizes catecholamines, are decreased in CKD. Here we show that renalase deficiency in a mouse knockout model causes increased plasma catecholamine levels and hypertension. Plasma blood urea nitrogen, creatinine, and aldosterone were unaffected. However, knockout mice had normal systolic function and mild ventricular hypertrophy but tolerated cardiac ischemia poorly and developed myocardial necrosis threefold more severe than that found in wild-type mice. Treatment with recombinant renalase completely rescued the cardiac phenotype. To gain insight into the mechanisms mediating this cardioprotective effect, we tested if gene deletion affected nitrate and glutathione metabolism, but found no differences between hearts of knockout and wild-type mice. The ratio of oxidized (NAD) to reduced (NADH) nicotinamide adenine dinucleotide in cardiac tissue, however, was significantly decreased in the hearts of renalase knockout mice, as was plasma NADH oxidase activity. *In vitro* studies confirmed that renalase metabolizes NADH and catecholamines. Thus, renalase plays an important role in cardiovascular pathology and its replacement may reduce cardiac complications in renalase-deficient states such as CKD.

Kidney International (2011) **79,** 853–860; doi:10.1038/ki.2010.488; published online 22 December 2010

KEYWORDS: amine oxidase; catecholamines; myocardial ischemia; NADH oxidase; renal failure; sympathetic nervous system

Correspondence: *Gary V. Desir, Section of Nephrology, Department of Medicine, Yale School of Medicine, P.O. Box 208029, New Haven, Connecticut 06520-8029, USA. E-mail: gary.desir@yale.edu*

[5]These authors contributed equally.

Received 21 April 2010; revised 6 September 2010; accepted 5 October 2010; published online 22 December 2010

Patients with chronic kidney disease (CKD) and end-stage renal disease develop coronary disease at a significantly higher rate than the general population.[1–4] Although traditional risk factors such as hypertension and hyperlipidemia are prevalent in CKD patients, they do not appear to fully account for the increased cardiac disease burden. It has been hypothesized that additional risk factors, including heightened sympathetic tone,[5–8] oxidative stress,[9] and arterial calcification, also contribute to the development of coronary artery disease in patients with CKD. For instance, plasma norepinephrine (NE) concentration predicts survival and incident cardiovascular events in patients with end-stage renal disease.[10] Sympathetic nerve activity, a proxy for sympathetic tone, and plasma catecholamine concentrations are elevated in patients with CKD stage 3 and above. The increase in plasma catecholamine concentrations appears to be because of an increase in their production, with spillover into the circulation, in addition to a reduction in their clearance.

We recently identified renalase, a novel flavin adenine dinucleotide (FAD)-dependent oxidase that participates in catecholamine metabolism. It is expressed in kidney, heart, and skeletal muscle, and secreted into the blood by the kidney.[11] Plasma renalase levels are decreased in patients with CKD and end-stage renal disease.[11,12] Plasma and kidney renalase levels are decreased in animals subjected to subtotal nephrectomy (5/6 Nx), thus providing a plausible explanation for the decreased catecholamine clearance observed in CKD.[13,14] A significant reduction in cardiac renalase levels was observed in the 5/6 Nx rat model.[13,14] This is particularly significant as the abnormal regulation of catecholamine metabolism contributes to the pathogenesis of left ventricular hypertrophy, ventricular arrhythmia, myocardial ischemia, and myocardial necrosis. We have further shown that renalase circulates as a proenzyme that lacks enzymatic activity under baseline conditions, and that is rapidly activated by elevated plasma catecholamines.[13] Additionally, two single-nucleotide polymorphisms in the renalase gene (rs2576178 GG genotype and rs2296545 CC) are associated with essential hypertension.[15] Interestingly, rs2296545 CC results in a conservative amino-acid change (glutamic to aspartic acid at amino acid

37 (E37D)) within the FAD-binding domain, and may affect the affinity of the binding site for FAD.

In this work, we used a knockout (KO) mouse model to examine the link between renalase deficiency, hypertension, and cardiovascular disease, and to gain insight into the mechanisms mediating renalase's cardioprotective effect.

RESULTS

Hypertension in renalase-deficient mice

Renalase plasma and tissue levels are decreased in animal models of CKD.[13,14] To test the hypothesis that renalase deficiency alone aggravates cardiac ischemic injury, we generated a renalase KO mouse by disrupting the renalase locus using homologous recombination. The targeting construct, shown in Figure 1a, deletes the promoter region and a large part of the coding region. Gene disruption was confirmed by genomic Southern blot (Figure 1b), PCR (Figure 1c), and western blot (Figure 1d). Renal function, assessed by blood urea nitrogen, serum creatinine, and urinary protein excretion, was unaffected by renalase deletion. Several noteworthy phenotypic characteristics are shown in Table 1. Blood pressure (BP) and heart rate were higher in anesthetized KO mice, and plasma aldosterone concentrations were not significantly different between wild-type (WT) and KO mice. As depicted in Figure 2, KO mice had higher plasma dopamine, epinephrine, and NE than WT mice. Adrenergic sensitivity was tested using TOCRIS A-61603 hydrobromide (St Louis, MO), a highly specific α-adrenergic receptor 1A/C agonist (αAR1A/C). Infusion of TOCRIS A-61603 at 0.2 μg/kg/min for 10 min increased BP significantly more in renalase KO mice than in WT (Figure 3a).

Ambulatory, telemetric BP measurements in conscious mice confirmed systolic and diastolic hypertension in the renalase KO, with a relatively greater elevation in diastolic pressure. Both KO and WT mice exhibit normal diurnal variations with peak pressure at ~2300 hours (Figure 3b). Systolic and diastolic pressures were significantly elevated in KO mice both during activity (Figure 3c) and at rest (Figure 3d). During the active period, renalase KO mice had a significantly higher heart rate than WT controls (627 ± 9 vs 582 ± 10 beats/min, $n = 7$, $P < 0.004$). Renalase KO mice had a normal left ejection fraction (in %, WT: 49.86 ± 5.15 vs KO: 53.24 ± 4.33, $n = 7$), and left ventricular hypertrophy as evidenced by increased left ventricular posterior wall thickness (in mm, WT: 0.79 ± 0.11 vs KO: 0.94 ± 0.09, $n = 7$, $P < 0.02$), and smaller left ventricular diameter (in mm, WT: 4.50 ± 0.07 vs KO: 4.23 ± 0.09, $n = 7$, $P < 0.05$), as assessed by transthoracic echocardiography. The left ventricular mass of KO mice was not significantly different from that of WT (in mg, WT: 167.06 ± 15.45 vs KO: 170.89 ± 8.09, $n = 7$, $P = 0.83$).

Renalase deficiency aggravates ischemic cardiac damage

To test if renalase deficiency modulates the response to cardiac ischemia, isolated perfused hearts were exposed to 15 min of global ischemia, followed by reperfusion for 90 min. Myocardial damage was assessed by triphenyltetrazolium staining of the heart. Viable tissue stains red, whereas damaged tissue fails to pick up the stain and appears pale. The degree of myocardial necrosis was qualitatively greater in renalase KO hearts (Figure 4a). Infarct size was quantified by computer-assisted planimetry (ImageJ 1.34s, National Institute of Health (NIH) Washington, DC), and as shown in Figure 4b, myocardial damage was threefold greater in KO mice. The cardioprotective effect of recombinant renalase was assessed by subjecting isolated KO mouse heart to global ischemia for 15 min, and then reperfusing for 90 min with buffer containing either renalase or glutathione synthase.

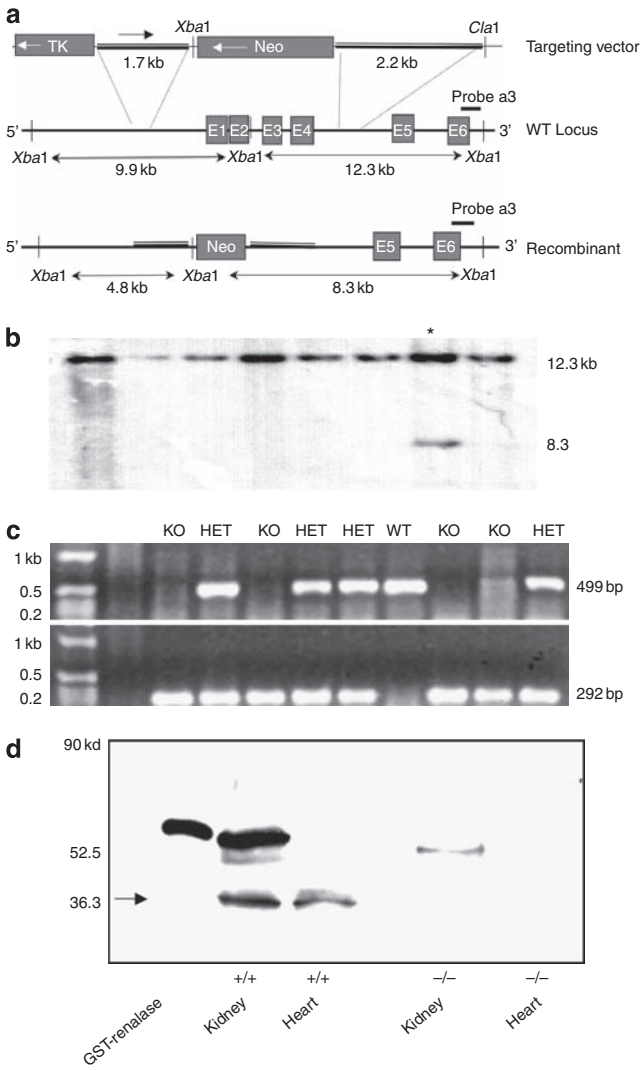

Figure 1 | Generation of renalase knockout (KO) mouse. (**a**) Targeting vector. E1–E6: exons 1–6; probe a3: 3′ end probe external to deleted region labels 12.3 and 8.3 kb *Xba*1 fragments in wild-type (WT) and KO, respectively. (**b**) Embryonic stem (ES) cell screen. Genomic Southern of ES cells using probe a3; *positive ES clone. (**c**) Mouse genotyping by PCR. The 499 bp band: WT allele; 292 bp band: KO allele. (**d**) Western blot of mouse tissue from WT and KO. The arrow indicates band of the expected size (37 kd) for renalase protein; 52.5 kd band in heart and kidney is nonspecific.

Table 1 | Comparison of renalase KO and WT mice

	WT	KO	*n*	*P*-value
Weight at 3 months (g)	30.8 ± 1.5	26.5 ± 1.2	20	<0.001
Activity index (AU)	4.54	6.01	4	<0.05
Anesthetized BP (mm Hg)				
Systolic	92.9 ± 3.4	103.2 ± 4.6	4	<0.04
Diastolic	61.7 ± 2.1	72.6 ± 2.0		<0.02
Mean	74.5 ± 2.3	85.2 ± 2.8		<0.02
Anesthetized heart rate (b.p.m.)	406.7 ± 38.1	509.3 ± 45.5	4	<0.05
Plasma aldosterone (pg/ml)	797.6 ± 82.9	796.6 ± 83.9	30	0.99
Plasma electrolytes (mEq/l)				
Sodium	144.9 ± 1.1	147.4 ± 0.9	10	0.06
Potassium	5.1 ± 0.1	4.7 ± 0.1		<0.001
Chloride	114.0 ± 0.5	117.0 ± 0.5		<0.001
Blood glucose (mg/dl)	238.6 ± 1.3	235.1 ± 0.9	10	0.43
Blood urea nitrogen (mg/dl)	17.2 ± 0.2	16.9 ± 0.3	10	0.26
Serum creatinine (mg/dl)	0.136 ± 0.012	0.130 ± 0.006	10	0.67
Urine protein/creatinine ratio	0.49 ± 0.21	0.45 ± 0.17	10	0.76
Reduced glutathione (nmol/mg protein)				
Plasma	0.292 ± 0.011	0.264 ± 0.014	6	0.44
Heart	2.94 ± 0.31	3.12 ± 0.29		0.59
Liver	8.77 ± 1.13	8.66 ± 1.26		0.63
Kidney	5.34 ± 0.41	5.34 ± 0.41		0.96
Nitrate+nitrite (μM)				
Plasma	3.12 ± 0.41	3.29 ± 0.45	6	0.37
Heart	18.32 ± 2.13	19.17 ± 2.37		0.33
Liver	6.11 ± 1.39	5.22 ± 0.94		0.42
Kidney	8.59 ± 1.67	7.91 ± 1.28		0.52

Abbreviations: AU, arbitrary units; BP, blood pressure; KO, knockout; WT, wild type. Higher activity index indicates greater levels of activity.

As shown in Figure 4c and d, compared with glutathione *S*-transferase, reperfusion with recombinant renalase was associated with a dramatic reduction in ischemic myocardial damage in renalase KO hearts.

Mechanisms of cardioprotection by renalase

As cardiac catecholamines, renalase substrates, increase by ~100-fold during cardiac ischemia *in vivo*,[16] it is likely that renalase's cardioprotective effect is mediated in part by its ability to metabolize excess NE and epinephrine. In support of this notion is the finding that in the isolated perfused heart model, NE administration caused a greater increase in the rate-pressure product in KO hearts compared with WT hearts (Figure 4e).

To search for additional mechanisms, we tested if renalase deletion altered the metabolism of non-amine compounds known to affect endothelial function and myocardial metabolism. Nitrate and nitrite concentrations in tissue and plasma did not differ significantly between WT and KO mice, suggesting that renalase deficiency does not affect nitrate metabolism. Similar levels of oxidized and reduced glutathione levels (plasma, kidney, heart, and liver) were measured in WT and renalase KO.

In silico analysis of renalase's secondary structure indicated that in addition to an FAD-binding motif, it also contained a putative NAD/NADH-binding site, which suggested that it might use NADH to reduce oxidized flavin, and therefore, function as a NADH oxidase.

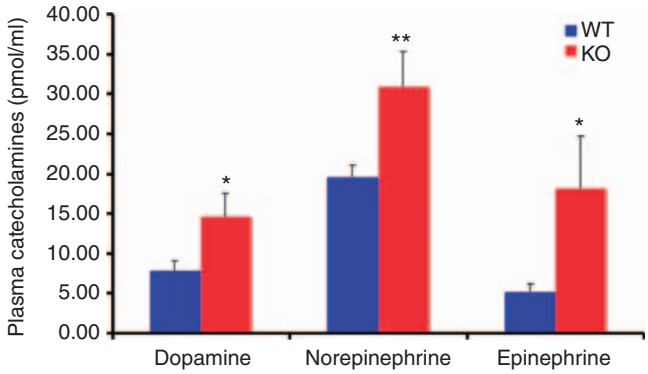

Figure 2 | Elevated plasma catecholamines in renalase knockout (KO). Plasma levels of dopamine, norepinephrine, and epinephrine in wild-type (WT, $n = 9$) and renalase knockout mice ($n = 11$) at 7 weeks of age. *$P < 0.05$; **$P < 0.01$.

We next tested whether renalase deficiency affected the cellular NAD/NADH ratio, and found a significant reduction in the ratio of oxidized to reduced nicotinamide adenine dinucleotide (NAD/NADH) in the cardiac tissue (Figure 5a). Plasma and tissue NADH oxidase activities were measured and found to be markedly decreased in renalase KO animals, supporting the hypothesis that renalase possesses NADH oxidase activity (Figure 5b). Recombinant renalase was tested *in vitro*, and was also found to have significant NADH oxidase activity in the absence of catecholamine substrates. Kinetic parameters, estimated by fitting initial velocity vs substrate concentration values to the Michaelis–Menten equation using nonlinear regression, were as follows: Km (NADH) = 15.23 ± 2.16 μM and Vmax = 15.27 ± 0.84 nmol/min per mg protein at 37 °C (Figure 5c).

DISCUSSION

Disruption of the renalase gene in mice causes hypertension in the absence of any measurable change in renal function. Continuous hemodynamic monitoring by telemetry revealed elevated BP in the KO animals during activity and at rest. The marked increase in diastolic pressure suggests heightened peripheral vasoconstriction in the renalase KO, as would be expected if renalase had a significant role in catecholamine metabolism. Under basal conditions, plasma catecholamines were significantly elevated in the renalase KO. Administration of a specific αAR1A/C agonist at a dose that minimally affected BP in WT animals caused a marked increased blood pressure in KO animals, indicating increased sensitivity to adrenergic stimulation. Furthermore, exogenous NE was associated with a greater hemodynamic effect in the KO heart, suggesting that renalase is likely to participate in the acute response to catecholamine release. This is in line with previous results indicating that an acute increase in plasma catecholamine concentrations rapidly activates circulating

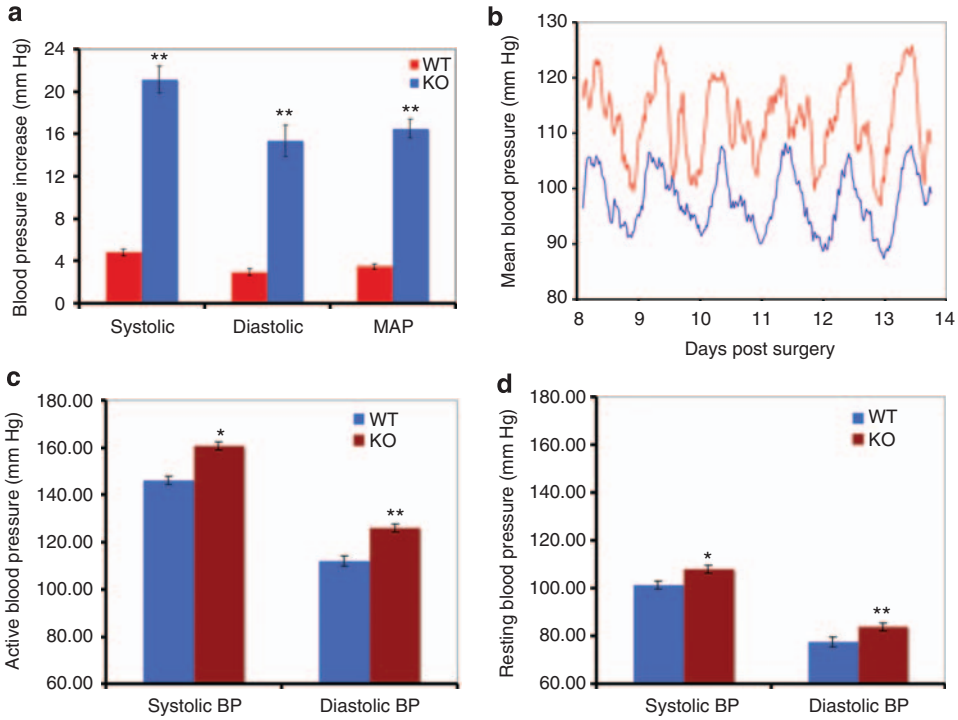

Figure 3 | Elevated blood pressure in renalase KO. (**a**) Increased sensitivity of α-adrenergic receptor 1A/C (αAR1A/C) in renalase KO. Increase in systolic pressure with infusion of αAR1A/C agonist, $n = 4$, $^{**}P < 0.0001$. (**b**) Ambulatory blood pressure in WT and KO. Transmitter surgically implanted at day 0; mean arterial pressure (MAP) measured continuously over a 7-day period. (**c**) Persistent blood pressure elevation in KO. BP, blood pressure; KO, renalase knockout; WT, wild-type littermates. Active period: BP measured between 1800 and 0600 hours; $^{*}P < 0.0001$, $^{**}P < 0.0002$, $n = 7$. (**d**) Resting pressure: BP measured between 0600 and 1800 hours; $^{*}P < 0.0002$, $^{**}P < 0.0002$, $n = 7$.

renalase and stimulates the secretion of the preformed enzyme,[13] and suggest that the increase in BP pressure observed in the KO mice is most likely of neurogenic origin.

Two single-nucleotide polymorphisms (rs2576178 GG genotype and rs2296545 CC) within the renalase gene are reported to be associated with essential hypertension.[15] Single-nucleotide polymorphism rs2296545 CC results in a conserved amino-acid change at amino acid 37 (glutamic to aspartic acid) within the FAD-binding domain (E37D). In monoamine oxidase-B, the FAD-binding domain consists of a β1 sheet-α1 helix–β2 sheet, beginning with a highly conserved GxGxxG motif, and ending with glutamic acid at position 34 (E34).[17,18] Site-directed mutagenesis of monoamine oxidase-B at position 34 revealed that a negative charge was absolutely required for function as E34A was nonfunctional and, surprisingly, although the negative charge was conserved in the E34D mutant, it was 93% less active that WT monoamine oxidase-B.[18] These data suggest that single-nucleotide polymorphism rs2296545 CC (E37D) may have profound effects on the enzymatic activity of renalase.

Renalase KO mice tolerate cardiac ischemia poorly, and when exposed to an ischemic insult develop myocardial necrosis that is threefold more severe than that seen in WT littermates. This difference cannot be explained by the mild hypertension and left ventricular hypertrophy that was observed in the renalase KO mice. Catecholamine levels increase markedly in the ischemic myocardium, and a 600-fold rise in norepinephrine levels has been documented by microdialysis in myocardial interstitial fluid of the ischemic pig heart.[16] Catecholamine release induced by ischemia does not depend on continuous cardiac innervation, and has been documented in the isolated perfused heart preparation.[19] In these studies, NE levels, measured from the coronary venous effluent, rose by 20-fold following 15 min of global ischemia. This corresponds to an increase in extracellular concentration of NE from 20 nM to 1 μM, a level that is well within the range known to cause myocardial necrosis.[20] It is, therefore, likely that the protective effect of exogenous renalase is due in part to its ability to metabolize catecholamines. Increased catecholamine levels are believed to aggravate cellular injury and predispose to malignant ventricular arrhythmias. Under basal conditions, the neuronal NE carrier (Uptake 1) transports NE into cells, and it appears to work in reverse during ischemia. Circulating renalase is known to be activated by catecholamine surges and could have a key role in limiting the accumulation of NE in myocardial interstitial fluid during ischemia, and further speed up its removal upon reperfusion. Renalase deficiency would be expected to impair NE disposal, which may explain the marked increased in ischemic cardiac injury observed in the renalase KO heart, and the cardioprotective effect of recombinant renalase.

A large proportion of the NE that accumulates in the myocardial interstitial fluid during ischemia is reported to

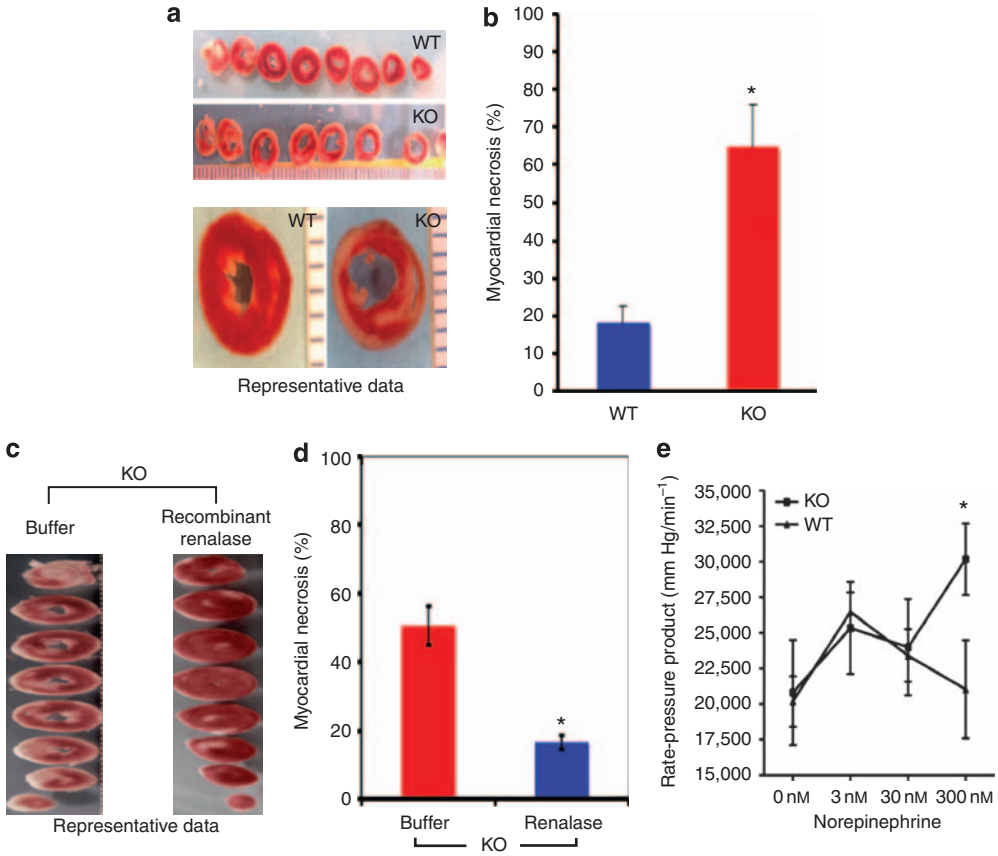

Figure 4 | Cardioprotective effect of renalase. (**a**) Increased ischemic myocardial damage in renalase KO. Triphenyltetrazolium (TTZ) stains of WT and KO hearts exposed to 15 min of global ischemia followed by 90 min of reperfusion; red stain indicates viable myocardium. KO, renalase knockout; WT, wild-type littermates. (**b**) Ischemic cardiac necrosis in WT and KO quantified using ImageJ. (**c**) Cardioprotective effect of recombinant renalase. TTZ stains of KO heart exposed to 15 min of global ischemia followed by 90 min of reperfusion with or without recombinant renalase; red stain indicates viable myocardium. (**d**) Recombinant renalase's cardioprotective effect quantified using ImageJ. *$P<0.002$, $n=4$. (**e**) Hemodynamic effect of norepinephrine in KO. *$P<0.04$, $n=4$.

come from cardiac nerves.[16] As our studies employed the isolated perfused heart model, which is denervated and has significantly lower catecholamine levels than the intact heart,[21] it is possible that catecholamine-independent mechanisms also have a role in mediating renalase's cardioprotective action. We found no evidence that renalase deficiency affected either nitrate or glutathione metabolism. Plasma and tissue nitrate and nitrite did not differ between KO and WT mice, suggesting that nitric oxide deficiency was an unlikely explanation of the KO's cardiovascular phenotype. Similarly, there were no changes in oxidized and reduced glutathione, which indicates that oxidative stress does not appear to be a major factor. However, renalase deficiency was associated with a significant decrease in the cellular NAD/NADH ratio. NAD is critically important in energy metabolism and electron transfer reactions. It modulates the activity of the NAD-dependent type III deacetylase surtuin-1 (SIRT1), which participates in metabolic homeostasis and cell survival pathways.[22] Elevated NAD levels enhance SIRT1 activity, resulting in the deacetylation and modulation of the activity of downstream targets such as transcription factors (FOXO1 and FOXO3a) and the peroxisome proliferator-activated receptor-c coactivator 1a. A decrease in the NAD/NADH ratio would be expected to aggravate myocardial injury during ischemia, and impair cardiac contractility during reperfusion.

We also found that plasma NADH oxidase activity was significantly lower in renalase KO, suggesting that plasma renalase contributes to the regulation of extracellular NAD (NADex) level. Although NAD's role as a coenzyme is well described, its involvement in cell signaling pathways has only recently been documented. NADex reportedly serves as a substrate for nucleotide-metabolizing enzymes, and as ligand for extra- and intra-cellular receptors and ion channels.[23] NADex regulates intracellular calcium signaling by interacting with specific cell surface receptors, such as the human P2Y11 purinergic receptor.[24,25] Although plasma NAD levels are difficult to measure accurately, they reportedly range between 0.1 and 4 μM. The conversion of NADH to NAD by plasma NADH oxidases is among the factors believed to contribute to the pool of NADex. The others include cell lysis, dephosphorylation of Cx43 hemichannels, and increased NAD release from cells.[26] Our data suggest that renalase may function as a plasma NADH oxidase, and it regulates NADex.

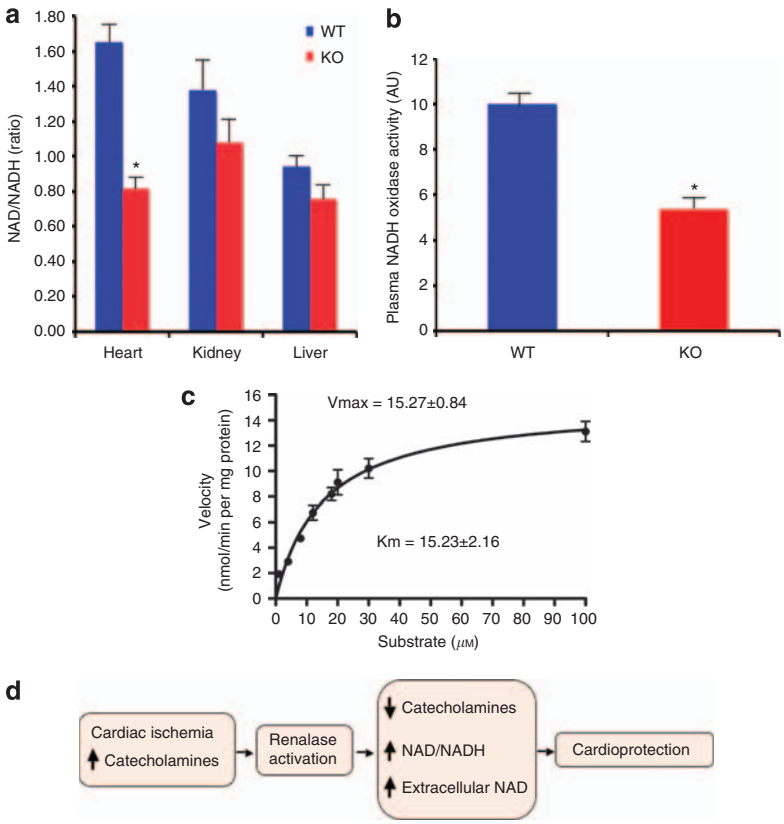

Figure 5 | Renalase modulates redox metabolism. (**a**) NAD + /NADH ratio in renalase KO. KO, renalase knockout; WT, wild-type littermates. *$P < 0.02$, $n = 6$. (**b**) Plasma oxidase activity in renalase KO; *$P < 0.008$, $n = 6$. (**c**) NADH oxidase activity of recombinant renalase. Km, substrate concentration needed to reach 1/2 maximal velocity; V, velocity; Vmax, maximal velocity. (**d**) Proposed mechanisms for renalase's cardioprotective effect. ↑ Indicates increase, and ↓ decrease.

Recombinant renalase metabolizes NADH in the absence of catecholamines, and its Vmax compares favorably with that of bacterial[27] and also mammalian NADH oxidases such as apoptosis-inducing factor.[28] Unlike apoptosis-inducing factor, it is specific for NADH and does not metabolize NADPH. Sera from patients suffering from cancer contain a capsaicin-sensitive NADH oxidase. The activity is reported to track with a protease-resistant 33.5 kd protein.[29] It is unclear if this protein also metabolizes NADPH, and its relationship to renalase is unknown at present.

In summary, renalase deficiency alone, in the absence of measurable changes in renal function, is associated with increased BP and susceptibility to ischemic myocardial damage. Renalase's cardioprotective effect appears to be mediated not only by its ability to metabolize catecholamines, but also by its effect of extracellular NAD and cellular redox metabolism (Figure 5d). Renalase levels in the plasma, kidney, and heart are decreased in CKD and end-stage renal disease. Taken together, these data support the notion that renalase has an important role in the regulation of BP and prevention of cardiac ischemic damage. We speculate that renalase deficiency increases the risk of cardiac arrhythmias and ischemic injury in patients with CKD, and that renalase replacement therapy may improve cardiovascular outcome in CKD.

MATERIALS AND METHODS

Generation of renalase KO mice

The animal studies were carried out according to the protocols approved by the institutional animal care and use committee of VA Connecticut Health Care System. The targeting vector was designed to delete a 10.048-kb region from the renalase gene, encompassing exons 1–4 and 4.465-kb upstream of the 5′ untranslated region (Figure 1a). To that end, we amplified 5′ and 3′ sequences (1.7 and 2.2 kb, respectively) flanking the region to be deleted by PCR from genomic DNA isolated from a 129Sv/J mouse, using the following primers: (F-5′-GAACGCGTCGACTTGTTTGAGTTCATAGTGGGG GAG-3′, R-5′-GAACGCGTCGACTACTGCTGCTGCTGTTCTTGG TGG-3′); (F-5′-AAGGAAAAAAGCGGCCGCTGTAGTCCTGGGTT GTGGGTTC-3′, R-5′-AAGGAAAAAAGCGGCCGCTGGGAAGGTG AAATGTCTCCG-3′). The amplified 1.7-kb 5′ DNA sequence was ligated into the *Sal*1 site of pEasy Flox downstream to the *neomycin* gene (*Neo*), whereas the 2.2-kb 3′ fragment was cloned into the *Not*1 site, upstream to the *Neo* gene. The targeting vector was linearized using *Cla*1, and 25 mg DNA was electroporated into 107 W9.5 embryonic stem cells. Cells were plated onto mitomycin C-treated embryonic fibroblasts and drug selection begun 24 h later. Embryonic stem cells were screened with probe a3 (849 bp PCR product: forward- 5′-CCTGACCCTTCTTCTGAACACACC-3′, reverse- 5′-TGACTGGCTTTTCTCTTTGAGTGG-3′), which detects different *Xba*1 fragment sizes in WT (12.3 kb) and recombinant (8.3 kb) renalase. Embryonic stem cells that underwent homologous recombination were selected and injected into C5BL/6 blastocysts,

and chimeric males were bred to C5BL/6 females. All mice were housed in specific pathogen-free conditions in accordance with the institutional animal care and use guidelines. WT and renalase KO mice were identified by PCR of tail genomic DNA (WT 499 bp product, F, 5′-AAATCCCCAGTTACTTATGGCTCC-3′, R, 5′-GAGA CAGTGACAGAGAGAAACCAGC-3′; renalase KO 292 bp product, F, 5′-AGGCTATTCGGCTATGACTGGG-3′, R, 5′-TGGATACTTTCTC GGCAGGAGC-3′). Gene deletion was confirmed by genomic Southern blot, reverse transcriptase-PCR of kidney tissue, and by western blotting of heart and kidney tissue. Renalase KO mice and control littermates used in these studies were F10–F12 offsprings obtained from B6/129 intercrosses.

Anesthetized BP measurements

BP were measured as previously described.[11,30] In brief, mice (20–40 g) were anesthetized with inactin (100 mg/kg), and a catheter (PE-50, Warner Instruments, Hamden, CT) was placed in the trachea for airway protection and in the left jugular vein (PE-10, Warner Instruments, Hamden, CT) for intravenous infusion of a maintenance fluid solution consisting of normal saline with 2.25% bovine albumin, at a rate of 12 µl per g body weight per hour. Core temperature was monitored using a rectal thermometer and body temperature was maintained at 37 °C using a heating pad. Arterial pressure and pulse were continuously monitored through a catheter inserted in the right carotid artery and connected to a pressure transducer (ADInstruments, Colorado Springs, CO). Hemodynamic recordings were digitized, stored, and analyzed using a PowerLab/8SP data acquisition system (ADIntruments). The mice were allowed 1 h to recover after completion of the surgical procedure, and BP and pulse were continuously recorded during the subsequent 30 min.

Ambulatory BP measurements

Ambulatory BP in mice was measured via telemetry using BP transducers (Data Sciences International, St Paul, MN, model TA11PA-C10) implanted into the carotid artery using sterile technique. The catheter of the transducer was inserted into the carotid artery and secured in place with silk suture. The body of the transducer was introduced into a subcutaneous pocket formed on the right lateral flank. Mice were allowed to recover for 7 days after surgery to regain their normal circadian rhythms before experiments were initiated. The animals were fed a standard (24%) protein diet (Purina, St Louis, MO) and synchronized to a 12/12 h light (0600 to 1800 hours) and dark (1800 to 0600 hours) cycle. The signals from the pressure sensor were converted and sent via the radio frequency transmitter to a telemetry receiver, connected to a BCM-100 consolidation matrix and a Dataquest IV acquisition system (Data Sciences International). Systolic and diastolic pressure and heart rate were continuously recorded at 5-min intervals, each reading representing the average BP during a 10-s sampling period.

In vivo measurement of cardiac function

Left ventricular size and function were assessed by echocardiography.[31] The tests were performed on WT and KO mice (3 months old) under light anesthesia with inhaled isoflurane. The echocardiographic data were collected in both short- and long-axis views at heart rates >400 b.p.m. A total of 5 measurements were made in each view (total 10 measurements) and averaged together to obtain functional and anatomical data. All imaging was performed using the Vevo770 system (VisualSonics, Toronto, Canada) with a 40 mHz probe.

Plasma aldosterone

Plasma aldosterone concentrations were determined using the DSL-8600 ACTIVE Aldosterone Coated-Tube Radioimmunoassay Kit according to the manufacturer's instructions (Diagnostic Systems Laboratories, Webster, TX).

Plasma catecholamine measurements

Catecholamines were assayed in plasma by high-pressure liquid chromatography with electrochemical detection, as previously described.[32] The lower limit of detection for dopamine, NE, and epinephrine ranged from 350 to 1000 fmol.

Synthesis of recombinant renalase protein

Recombinant renalase (37E isoform) was synthesized as previously described.[11] Briefly, the renalase coding region was amplified by PCR and cloned into the pGEX-4T (Promega, Madison, WI) in frame with a glutathione *S*-transferase tag (26 kDa) at the N-terminus. *Escherichia coli* BL21 were transformed and grown at 37 °C for 16 h with 0.1 µM FAD. Isopropyl β-D-1-thiogalactopyranoside (0.5 mmol/l) was added for the last 3.5 h of culture. The renalase fusion protein was purified using Glutathione Sepharose. Untagged recombinant renalase was also synthesized in *E. coli*, purified, and refolded.

Cardiac ischemia/reperfusion

Isolated perfused hearts were examined by the Langendorff method using a Radnoti isolated perfused heart System (model 120103EZ, Monrovia, CA).[33] Hearts were excised from adult mice (renalase KO and WT littermates) that had been anesthetized, weighed, and heparinized (500 U/100 g body weight), and immediately placed in ice-cold buffer solution. The aorta was cannulated on a 20-gauge Luer stub adapter (Warner Instruments, Hamden, CT) with a stainless steel shaft and perfused with physiological buffer, at a flow rate of 1.5 ml/min. An incision was made at the root of the pulmonary artery to drain coronary effluent. A constant-flow pump provided coronary perfusion at a rate of $\sim$2 ml/min through the aorta. The coronary perfusate consisted of (in mmol/l) 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 23 $NaHCO_3$, 10.0 dextrose, and 0.3 EDTA, gassed with 95% O_2/5% CO_2 and adjusted to pH of 7.4. The hearts were then subjected to no-flow global ischemia for 15 min, and then reperfused for 90 min. Left ventricular function was measured using a fluid-filled latex balloon containing a catheter-tip transducer (model SPR-671; Millar, Houston, TX) inserted into the left ventricle. The distal end of the balloon system was connected via pressure transducer to PowerLab ADInstruments, and intraventricular pressure was digitally recorded. Infarction size was assessed using triphenyltetrazolium staining. At the end of the experimental procedure, a 10% (wt/vol) solution of triphenyltetrazolium chloride in phosphate buffer was infused into the coronary vasculature through a side arm of the aortic cannula. Once the heart had been discolored (triphenyltetrazolium stains viable myocardium deep red), the heart was perfused with 4% formalin. The heart was sectioned into 1 mm thick slices, which were illuminated and photographed. Myocardial infarct size was assessed by computer-assisted planimetry (ImageJ 1.34s) of triphenyltetrazolium-stained sections.

Nitrate and nitrite measurements

Plasma and tissue nitrate and nitrite were measured using the Nitrate/Nitrite calorimetric assay kit, and according to the manufacturer's instructions (Cayman Chemical Company, Ann Harbor, MI).

Glutathione levels

Reduced glutathione and oxidized glutathione were measured in plasma and tissue using the Glutathione Assay Kit, and according to the manufacturer's instructions (BioVision Research Products, Mountain View, CA).

NAD and NADH measurements

Tissue NAD + and NADH concentrations in KO and WT mice were determined by calorimetry using the EnzyChrom NAD + /NADH assay kit, and according to the manufacturer's protocol (Bioassay Systems, Hayward, CA).

NADH oxidase activity

The assay buffer contained 50 mmol/l potassium phosphate, pH 7.5, and 1 mmol/l EDTA. NADH was made fresh and added to a final concentration ranging from 50 to 1000 μM. The reactions were initiated by adding plasma (40 μl) or recombinant renalase (6–30 μg) to 200 μl/ml of assay buffer in 96-well plate cuvettes (0.6-cm path length). Absorbance at 340 nM was measured in a plate reader at 25 or 37 °C, and recorded every 4 min for 60 min. The amount of NADH oxidized to NAD was calculated from the decrease in absorbance at 340 nM using a molar extinction coefficient of 0.00622 μmol/cm at 340 nm. To estimate kinetic parameters (Km and Vmax), initial velocity was plotted against substrate concentration, and the data were fitted to the Michaelis–Menten equation using nonlinear regression (GraphPad Prism, GraphPad Software, La Jolla, CA).

Western blot analysis

Studies were carried out as previously described using a polyclonal anti-renalase antibody.[11]

Statistical analysis

Standard unpaired Student's *t*-tests were used for group comparisons at equivalent periods. All data are means ± s.e., and $P < 0.05$ was accepted as a statistically significant difference.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

RAF is an investigator of the Howard Hughes Medical Institute.

Disclaimer

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.

REFERENCES

1. Anavekar NS, McMurray JJ, Velazquez EJ *et al.* Relation between renal dysfunction and cardiovascular outcomes after myocardial infarction. *N Engl J Med* 2004; **351**: 1285–1295.
2. Go AS, Chertow GM, Fan D *et al.* Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004; **351**: 1296–1305.
3. Hostetter TH. Chronic kidney disease predicts cardiovascular disease. *N Engl J Med* 2004; **351**: 1344–1346.
4. Coca SG, Peixoto AJ, Garg AX *et al.* The prognostic importance of a small acute decrement in kidney function in hospitalized patients: a systematic review and meta-analysis. *Am J Kidney Dis* 2007; **50**: 712–720.
5. Hausberg M, Kosch M, Harmelink P *et al.* Sympathetic nerve activity in end-stage renal disease. *Circulation* 2002; **106**: 1974–1979.
6. Klein IH, Ligtenberg G, Neumann J *et al.* Sympathetic nerve activity is inappropriately increased in chronic renal disease. *J Am Soc Nephrol* 2003; **14**: 3239–3244.
7. Joles JA, Koomans HA. Causes and consequences of increased sympathetic activity in renal disease. *Hypertension* 2004; **43**: 699–706.
8. Koomans HA, Blankestijn PJ, Joles JA. Sympathetic hyperactivity in chronic renal failure: a wake-up call. *J Am Soc Nephrol* 2004; **15**: 524–537.
9. Oberg BP, McMenamin E, Lucas FL *et al.* Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 2004; **65**: 1009–1016.
10. Zoccali C, Mallamaci F, Parlongo S *et al.* Plasma norepinephrine predicts survival and incident cardiovascular events in patients with end-stage renal disease. *Circulation* 2002; **105**: 1354–1359.
11. Xu J, Li G, Wang P *et al.* Renalase is a novel, soluble monoamine oxidase that regulates cardiac function and blood pressure. *J Clin Invest* 2005; **115**: 1275–1280.
12. Feng W, Nian-song W, Tao X *et al.* The cloning and expression of renalase and the preparation of its monoclonal antibody. *J Shanghai Jiaotong Univ (Sci)* 2009; **14**: 376–379.
13. Li G, Xu J, Wang P *et al.* Catecholamines regulate the activity, secretion, and synthesis of renalase. *Circulation* 2008; **117**: 1277–1282.
14. Ghosh SS, Krieg RJ, Sica DA *et al.* Cardiac hypertrophy in neonatal nephrectomized rats: the role of the sympathetic nervous system. *Pediatr Nephrol* 2009; **24**: 367–377.
15. Zhao Q, Fan Z, He J *et al.* Renalase gene is a novel susceptibility gene for essential hypertension: a two-stage association study in northern Han Chinese population. *J Mol Med* 2007; **85**: 877–885.
16. Lameris TW, de Zeeuw S, Alberts G *et al.* Time course and mechanism of myocardial catecholamine release during transient ischemia in vivo. *Circulation* 2000; **101**: 2645–2650.
17. Edmondson DE, Binda C, Mattevi A. The FAD binding sites of human monoamine oxidases A and B. *Neurotoxicology* 2004; **25**: 63–72.
18. Kwan SW, Lewis DA, Zhou BP *et al.* Characterization of a dinucleotide-binding site in monoamine oxidase B by site-directed mutagenesis. *Arch Biochem Biophys* 1995; **316**: 385–391.
19. Schomig A, Dart A, Dietz R *et al.* Release of endogenous catecholamines in the ischemic myocardium of the rat. Part A: Locally mediated release. *Circ Res* 1984; **55**: 689–701.
20. Waldenstrom AP, Hjalmarson AC, Thornell L. A possible role of noradrenaline in the development of myocardial infarction: an experimental study in the isolated rat heart. *Am Heart J* 1978; **95**: 43–51.
21. Cooper T, Willman VL, Jellinek M *et al.* Heart autotransplantation: effect on myocardial catecholamine and histamine. *Science* 1962; **138**: 40–41.
22. Canto C, Gerhart-Hines Z, Feige JN *et al.* AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* 2009; **458**: 1056–1060.
23. Koch-Nolte F, Haag F, Guse AH *et al.* Emerging roles of NAD+ and its metabolites in cell signaling. *Sci Signal* 2009; **2**: mr1.
24. Bruzzone S, Moreschi I, Guida L *et al.* Extracellular NAD+ regulates intracellular calcium levels and induces activation of human granulocytes. *Biochem J* 2006; **393**: 697–704.
25. Moreschi I, Bruzzone S, Nicholas RA *et al.* Extracellular NAD+ is an agonist of the human P2Y11 purinergic receptor in human granulocytes. *J Biol Chem* 2006; **281**: 31419–31429.
26. Bruzzone S, Guida L, Zocchi E *et al.* Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells. *FASEB J* 2001; **15**: 10–12.
27. Arcari P, Masullo L, Masullo M *et al.* A NAD(P)H oxidase isolated from the archaeon Sulfolobus solfataricus is not homologous with another NADH oxidase present in the same microorganism. Biochemical characterization of the enzyme and cloning of the encoding gene. *J Biol Chem* 2000; **275**: 895–900.
28. Miramar MD, Costantini P, Ravagnan L *et al.* NADH oxidase activity of mitochondrial apoptosis-inducing factor. *J Biol Chem* 2001; **276**: 16391–16398.
29. Chueh PJ, Morre DJ, Wilkinson FE *et al.* A 33.5-kDa heat- and protease-resistant NADH oxidase inhibited by capsaicin from sera of cancer patients. *Arch Biochem Biophys* 1997; **342**: 38–47.
30. Xu J, Li G, Wang P *et al.* Catecholamines regulate the activity, secretion and synthesis of renalase. *Circulation* 2008; **117**: 1277–1282.
31. Park SY, Cho YR, Kim HJ *et al.* Unraveling the temporal pattern of diet-induced insulin resistance in individual organs and cardiac dysfunction in C57BL/6 mice. *Diabetes* 2005; **54**: 3530–3540.
32. Soares-da-Silva P, Pestana M, Fernandes MH. Involvement of tubular sodium in the formation of dopamine in the human renal cortex. *J Am Soc Nephrol* 1993; **3**: 1591–1599.
33. Russell III RR, Li J, Coven DL *et al.* AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J Clin Invest* 2004; **114**: 495–503.