The serum protein renalase reduces injury in experimental pancreatitis

Acute pancreatitis is a disease associated with inflammation and tissue damage. One protein that protects against acute injury, including ischemic injury to both the kidney and heart, is renalase, which is secreted into the blood by the kidney and other tissues. However, whether renalase reduces acute injury associated with pancreatitis is unknown. Here, we used both in vitro and in vivo murine models of acute pancreatitis to study renalase’s effects on this condition. In isolated pancreatic lobules, pretreatment with recombinant human renalase (rRNLS) blocked zymogen activation caused by cerulein, carbachol, and a bile acid. Renalase also blocked cerulein-induced cell injury and histological changes. In the in vivo cerulein model of pancreatitis, genetic deletion of renalase resulted in more severe disease, and administering rRNLS to cerulein-exposed WT mice after pancreatitis onset was protective. Because pathological increases in acinar cell cytosolic calcium levels are central to the initiation of acute pancreatitis, we also investigated whether rRNLS could function through its binding protein, plasma membrane calcium ATPase 4b (PMCA4b), which excretes calcium from cells. We found that PMCA4b is expressed in both murine and human acinar cells and that a PMCA4b-selective inhibitor worsens pancreatitis-induced injury and blocks the protective effects of rRNLS. These findings suggest that renalase is a protective plasma protein that reduces acinar cell injury through a plasma membrane calcium ATPase. Because exogenous rRNLS reduces the severity of acute pancreatitis, it has potential as a therapeutic agent.

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3 The abbreviations used are: RNLS, renalase; rRNLS, recombinant human renalase; PMCA, plasma membrane calcium ATPase; CER, cerulein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TLCS, tauroliothiocholic acid sulfate; IF, immunofluorescence.
the mouse. Cerulein (CER) is an orthologue of the mammalian hormone cholecystokinin. When given at concentrations 10-100-fold greater than physiologic levels, cerulein reproducibly causes an edematous and non-lethal form of acute pancreatitis. In acinar cells, we found that rRNLS reduced cerulein-induced injury. In a mouse with genetic deletion of RNLS, cerulein-induced pancreatitis was worse than that seen in wild-type (WT) mice. Administering exogenous rRNLS after the induction of cerulein pancreatitis reduced disease severity. These protective effects of renalase are likely related, at least in part, to activation of plasma membrane calcium ATPase. These results provide a basis for further investigation into RNLS as a potential treatment for pancreatitis.

**Results**

**Renalase pretreatment decreases pancreatitis responses in isolated pancreatic tissue**

The potential protective effects of renalase were first examined in pancreatic lobules exposed to agents that elicited early pancreatitis responses in acinar cells. Lobules treated with secretagogues showed variation in both basal and stimulated zymogen activation among experiments but had the same pattern of activation. For this reason, data are presented as -fold versus maximal response. We observed that the effects of rRNLS on CER-stimulated zymogen activation were concentration-dependent, inhibiting both trypsinogen (Fig. 1A) and chymotrypsinogen (Fig. 1B) activities were assayed. *, p < 0.05 compared with cerulein, carbachol, or TLCS alone. For cerulein, n = 7 studies (n = 3 studies for 50 μg/ml RNLS); for carbachol, n = 4 studies; for TLCS, n = 5 studies. Values represent the mean, and error bars represent the S.E.
provide evidence that RNLS can directly act on the pancreatic acinar cell to reduce pancreatitis injury. We next examined the effects of pancreatitis on plasma RNLS levels.

**Induction of pancreatitis decreases serum renalase levels, which return to baseline prior to recovery from pancreatitis**

Within 15 min of inducing CER pancreatitis, plasma RNLS levels were reduced; the lowest value was reached by 1 h (Fig. 3A). Levels remained low during the entire 6-h course of treatment but returned to control levels 6 h after CER treatment stopped (at 12 h). Markers of pancreatitis such as serum amylase (Fig. 3B) and tissue edema (Fig. 3C) returned to control levels by 24 h after the initiation of pancreatitis. Thus, return of plasma RNLS to control values appears to precede other parameters of pancreatitis recovery.

**Genetic deletion of renalase (KO) increases pancreatitis in vivo**

The effects of endogenous RNLS in control conditions and on the severity of CER pancreatitis (7 h after induction) were examined in mice with a pan-deletion of RNLS. This genetic model was developed in our laboratory and its primary phenotype is mild hypertension (8). No overt differences were seen in baseline responses (data not shown). After induction of CER pancreatitis, we found that RNLS deletion did not change either

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**Figure 2. RNLS reduces injury in pancreatic lobules.** Pancreatic lobules were preincubated with RNLS (25 μg/ml) for 30 min prior to CER (100 nM) administration to induce pancreatitis responses. Markers of cellular injury were assayed. A, MTT conversion. B, panels 1–4, pancreatic histology (H&E staining; photos taken at 400×). *, p < 0.05 versus control; #, p < 0.05 versus CER alone. For MTT, n = 4 studies; for histology, n = 3 studies. Values represent the mean, and error bars represent the S.E.
tissue edema (percent wet weight) or serum amylase levels (data not shown). Some histological features of CER-induced pancreatitis were worse in RNLS knock-out mice, including edema and macrophage infiltration, but not neutrophil infiltration or vacuolization, and the number of pyknotic nuclei decreased (Fig. 4). These findings suggest that genetic deletion of RNLS worsens select early parameters of CER-induced pancreatitis compared with WT. We next determined whether exogenous RNLS could reduce the severity of CER-induced pancreatitis.

Effects of renalase after the onset pancreatitis

To better reflect the clinical presentation in which most pancreatitis patients are seen after disease onset, rRNLS was given after disease onset in our experimental model. rRNLS administration did not affect baseline parameters such as tissue edema (by percent wet weight) or serum amylase (data not shown). However, CER pancreatitis was much less severe histologically (Fig. 5). This included less morphologic edema (Fig. 5E), fewer vacuoles (Fig. 5F), and a significant decrease in neutrophil infiltration (Fig. 5H). Together, these findings suggest that RNLS has protective effects in cellular and in vivo models of acute experimental pancreatitis. Because other studies from our group have suggested that RNLS may act through a plasma membrane calcium ATPase, we investigated whether this could also be a target in the pancreas (7).

PMCA gene expression in pancreatic acinar cells

We recently used photoaffinity cross-linking to identify plasma membrane RNLS-binding proteins; the PMCA type 1 and 4b isoforms were identified (7). Genetic down-regulation of PMCA4b was shown to eliminate the protective effect of RNLS in cultured cells (7). To determine whether such transporters were encoded for in the pancreatic acinar cell, we used polymerase chain reaction (PCR) analysis and PMCA isoform-specific primers. Mouse brain, which contains all major PMCA isoforms, was used as a positive control. Murine pancreatic acinar cells showed transcripts for PMCA1, -2, and -4. PMCA4 has two splice variants, PMCA4a and PMCA4b; only PMCA4b was found in acinar cells (Fig. 6A). In human pancreatic acinar cells, PMCA1 and -4b were highly expressed (Fig. 6B). We next examined the localization of PMCA4 in acinar cells.

Localization of PMCA4b in pancreatic lobules

To determine the distribution of PMCA4b in pancreatic tissue, we double labeled control pancreas with an antibody specific for PMCA4b (JA-3) and phalloidin, which labels apical F-actin bundles in the acinar cell. We found that PMCA4b primarily had a predominately basal distribution and did not colocalize with phalloidin staining (Fig. 6). This distribution is consistent with a basolateral localization of the transporter in most other epithelial cells, although an apical distribution has been reported in the parotid acinar cell (9, 10). Whether PMCA could mediate the effects of RNLS in the acinar cell was next investigated using an inhibitor of PMCA and examining the effects of RNLS on secretagogue-stimulated zymogen activation and cellular injury.
A PMCA inhibitor blocks the protective effect of renalase on secretagogue-stimulated pancreatitis in pancreatic lobules

To determine whether PMCA was involved in the protective effects of RNLS, we used caloxin 1b1, a peptide inhibitor that exhibits selectivity for PMCA4a and PMCA4b. We preincubated pancreatic lobules with caloxin 1b1 (100 μM) for 30 min prior to the addition of RNLS followed by CER stimulation. As shown in Fig. 7, we found that caloxin 1b1 pretreatment had no significant effect on basal zymogen activation (Fig. 7, A and B) or amylase secretion (Fig. 7C). However, it blocked the protective effect of renalase on CER (100 nM)-stimulated trypsinogen (Fig. 7A) and chymotrypsin (Fig. 7B) activation. Caloxin 1b1 pretreatment reversed the decrease in CER-stimulated amylase secretion seen with RNLS (Fig. 7C). Interestingly, caloxin...
tended to enhance CER (100 nM)-stimulated zymogen activation when RNLS was not present. To further examine the effect of caloxin 1b1 on zymogen activation, we pretreated lobules with caloxin with or without various concentrations of CER (Fig. 8, A and B). Caloxin 1b1 tended to enhance both basal and CER-stimulated trypsin activation (Fig. 8A). Although the same pattern was seen for chymotrypsin, the changes were not significant (Fig. 8B). Similar results for caloxin 1b1 sensitization were seen with trypsinogen (Fig. 8C) and chymotrypsinogen (Fig. 8D) activation in response to carbachol. The effects of...

Figure 5. RNLS administration after the induction of cerulein pancreatitis decreases histologic changes associated with cerulein pancreatitis. WT mice were given six hourly injections of either PBS as a control or cerulein (50 μg/kg) to induce pancreatitis. Concurrent with the third cerulein injection, RNLS (4 mg/kg) was given to half of the animals in each group, and PBS was given to the other half. Tissues were fixed in buffered formalin and stained with H&E. A, control (CTL). B, cerulein. C, RNLS (REN) alone. D, RNLS after cerulein. Stained slides were examined, and random fields were photographed at 400×. Sections were scored in a blinded manner for histologic markers of pancreatitis, including edema (E), intracellular vacuoles (F), pyknotic nuclei and apoptotic bodies (G), neutrophils (H), and macrophages (I). *, p < 0.05 compared with cerulein alone. n = 4 for each treatment group. Values represent the mean, and error bars represent the S.E.
RNLS on cell injury were then evaluated. As shown in Fig. 9, pretreatment with caloxin 1b1 blocked the protective effects of RNLS after CER hyperstimulation as measured by the MTT assay of metabolic function. Although caloxin alone enhanced cell injury, it did not increase injury induced by supraphysiologic CER. Caloxin alone had no effect on injury associated with lower doses of CER (data not shown). Lastly, caloxin alone did not cause intracellular vacuole formation as is seen in CER treatment (data not shown). Together, these studies suggest that the protective effects of RNLS on CER-induced acinar cell injury are mediated by a PMCA. Based on our previous studies using affinity cross-linking to identify RNLS-binding proteins and knockdown studies in other cell types, these effects are likely mediated by PMCA4b.

Discussion

Here, we examine whether RNLS, a plasma protein with protective effects in models of acute renal and cardiac injury (11, 12), could decrease injury in murine models of acute pancreatitis and whether it can act directly on the pancreatic acinar cell. Using the standard cellular and in vivo models of cerulein-induced acute pancreatitis, we observed a protective effect of RNLS. This included the effects of rRNLS on injuries generated by CER, carbachol, and bile acids on isolated groups of acinar cells. Global
deletion of RNLS in mice also resulted in more injury in the CER model, whereas administration of recombinant RNLS to WT mice reduced the severity of acute pancreatitis. When pancreatitis was induced by CER treatment, there was a rapid decrease in plasma renalase levels, reaching a nadir by 1 h. A low level of plasma RNLS persisted until 6 h when CER treatment ended; after 12 h, plasma RNLS levels returned to or exceeded baseline levels. The recovery of plasma renalase at 12 h preceded the resolution of pancreatitis, including increased pancreatic edema and serum amylase, which only normalized 24 h after the onset of injury. It may be that the return of plasma renalase levels to normal played a role in disease resolution.

Circulating RNLS may target multiple resident cell types within the pancreas, including endothelial cells, duct cells, islet
Akt-dependent mechanisms that preserve ATP levels as well through activation of PMCA (17). This study concluded that, under control conditions, Ca\textsuperscript{2+} signaling and PMCA activity were observed (15). The authors suggested that dysregulated RNLS signaling could bias macrophages to an M2-like phenotype (13). We have also found that RNLS suppresses inflammasome activity in stimulated macrophages.\textsuperscript{4} These findings are consistent with RNLS acting to suppress acute inflammatory responses. The plasma membrane ATPase PMCA4b has been identified as a receptor for renalase (7). Macrophages have been found to express PMCA4 (14). Studies are underway to examine the role of RNLS in macrophage responses in pancreatitis; we believe that RNLS will be found to have a role in modulating acute inflammation as well as resolution of this disease.

Previous studies have implicated PMCA in acute pancreatic acinar cell injury. In a study of oxidant stress induced by H\textsubscript{2}O\textsubscript{2}, complex concentration-dependent effects on acinar cell Ca\textsuperscript{2+} signaling and PMCA activity were observed (15). The authors concluded that, under control conditions, Ca\textsuperscript{2+} extrusion through PMCA was the major pathway for lowering cytosolic Ca\textsuperscript{2+}. Noting that insulin appears to have a protective role in models of exocrine pancreatic injury, its effects were examined in the setting of acinar cell oxidant-induced injury (16). In this study, insulin was found to reduce acinar cell injury, increase PMCA-dependent Ca\textsuperscript{2+} extrusion, and reverse oxidant-dependent PMCA inhibition. Finally, in a pancreatic acinar cell model thought to simulate the oxidative conditions that cause alcohol- and fatty acid–related acute pancreatitis, insulin was shown to be protective and reduce Ca\textsuperscript{2+} overload through activation of PMCA (17). This study concluded that the effects of insulin on PMCA activities were through PI3K/ Akt–dependent mechanisms that preserve ATP levels as well as unidentified PI3K/Akt–independent mechanisms. Together with the findings of this study, we conclude that PMCA activation can reduce acinar cell pancreatitis injury (7). However, the present study provides three important advances. First, unlike insulin, it reports that activation of PMCA by a ligand that likely binds its extracellular domain (RNLS) can be protective. Second, it demonstrates the potential benefits of this ligand using an in vivo model of acute pancreatitis. Finally, by demonstrating that genetic deletion of RNLS results in more severe experimental pancreatitis, it suggests a role for the endogenous protein in modulating injury. This is further supported by our results showing that inhibition of PMCA4 enhanced secretagogue-stimulatedzymogen activation even at subpathologic concentrations.

PMCA4s are a widely distributed protein family with four major members that each modulates cellular calcium efflux as well as other signaling pathways (18). PMCA activities and functions are regulated by the lipid environment and subcellular distribution. Variations in isoforms mediate interactions between different PMCA isoforms and distinct scaffolding and signaling pathways. For example, PMCA4b can suppress neuronal NOS and NO generation (19). Work from our laboratory, based on siRNA knockdown, has shown that PMCA4b isoform mediates the protective effects of RNLS in other cell types (7). Here, we show evidence that, in the murine pancreatic acinar cell, PMCA4 immunoreactivity is distributed primarily to the cell’s basolateral domain. This finding is consistent with what is found in human pancreas (20). Based on this and the presence of PMCA4b mRNA in murine and human pancreatic acinar cells, we believe that it is also likely an effector for RNLS in pancreatic acinar cells. However, because we also detected PMCA1 mRNA in acinar cells, we cannot exclude it as an RNLS target in this system. We also found that RNLS prevented acinar cell injury and that chemical inhibition of PMCA with caloxin 1b1 blocked this protective effect of RNLS on cell injury. In addition, we found that treatment with caloxin 1b1 enhanced both CER- and carbachol-stimulatedzymogen activation. This suggests that activation of PMCA4 by RNLS or other mechanisms can reduce acinar cell injury. This, along with our observation that plasma levels of RNLS dramatically fall with CER treatment, suggests that the loss of circulating RNLS may modulate disease severity. PMCA activation can affect calcium efflux as well as other signaling pathways, including Akt, ERK, NO, and CAMP levels (4). Which of these pathways is linked to the protective effects of RNLS and whether RNLS has important interactions with other plasma membrane proteins are important topics for future study.

In vivo studies using the CER model of acute pancreatitis showed that administration of exogenous recombinant RNLS reduced the severity of cerulein pancreatitis when given after disease onset. Although we demonstrated that RNLS can reduce acute injury to acinar cells, its effectiveness when given after disease onset could reflect its action on the acinar cell as well as other cell types, including inflammatory cells. For example, our published and preliminary studies suggest that RNLS can suppress innate immune responses in macrophages, a cell population that has been shown to mediate injury in acute pancreatitis (6).\textsuperscript{4}
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Observations made in these animal and cellular models could be relevant to human disease. For example, that the RNLS−/− mouse develops more severe pancreatitis suggests that deficiency of this protein in humans could increase the severity of acute injury. In this context, RNLS was discovered as a circulating protein that largely disappears in subjects with renal failure. Renal failure is associated with a greater risk of both developing acute pancreatitis and experiencing more severe disease. It is possible that RNLS deficiency contributes to the pancreatitis risk in renal failure. The reduced RNLS levels early in the course of experimental pancreatitis could also account for the benefit of giving exogenous RNLS. Functional polymorphisms in RNLS have been linked to hypertension in humans. It will be of interest to know whether similar variations in this protein can modulate the severity of acute injury, including acute pancreatitis.

In summary, we have shown that the recently described plasma protein RNLS reduces acute pancreatitis injury in cellular and in vivo murine models of this disease and that genetic deletion of RNLS led to more severe pancreatitis. An important target for this protective effect is likely the activation of PMCA4b on the pancreatic acinar cell. Our results suggest that RNLS may act through PMCA4 to prevent cell injury associated with pancreatitis and provide initial data suggesting that RNLS could serve as a treatment of acute disease.

Experimental procedures

Ethics statement

All experiments and procedures using animals were approved by the Veterans Administration Institutional Animal Care and Use Committee, West Haven, CT (Veterans Administration Public Health and Safety, Office of Laboratory Animal Welfare Assurance Number A4363-01).

Animals used

All wild-type animals used were male C57/BL6 mice weighing approximately 25 g. The renalase KO mice are on a C57/BL6 background, and both male and female animals used due to availability (8).

Preparation and isolation of recombinant renalase

The gene encoding human renalase1 (hRNLS) was purchased from Enzymax and cloned into pET-28a(+) expression vector (Novagen) to incorporate an N-terminal His tag. BL21 cells (Novagen) were transected and grown in LB medium containing 100 μg/ml kanamycin. Once cultures reached an OD of 1, isopropyl-1-thio-β-D-galactopyranoside (0.1 mM final) was added to induce protein production. Cultures were grown overnight with shaking at room temperature to avoid the formation of inclusion bodies, and the pancreas was removed. For lobules, pancreatic fragments (∼5-mm diameter) were mechanically separated and washed with incubation buffer (10 mM Hepes (pH 7.4), 95 mM NaCl, 4.7 mM KCl, 0.6 mM MgCl2, 1 mM Na2HPO4, 10 mM glucose, 2 mM glutamine, 0.1% bovine serum albumin, and 1× minimum essential medium amino acids (Gibco)). One to two lobules were then placed into each well of a 24-well Falcon tissue culture plate (BD Biosciences) containing 0.5 ml of incubation medium. For pancreatic acini, the removed pancreas was minced and processed by mechanical disruption and collagenase digestion as described (21). Acini were placed in the incubation buffer above and processed for pancreatitis responses as described (21). All reagents were purchased from Sigma unless otherwise noted.

Induction of pancreatitis in pancreatic lobules and pretreatment with renalase and/or caloxin 1b1

Lobules were pretreated with RNLS (5–25 μg/ml) prior to the induction of pancreatitis by one of the following secretagogues for 30 min to 2 h (depending on the experiment) with either CER (0.1–100 nM), carbachol (1 μM–1 mM), or tauro-lithocholic acid sulfate (TLCS; 500 μM) to induce pancreatitis responses. RNLS (1–25 μg/ml of medium) was added to appropriate wells 30 min prior to the addition of CER, carbachol, or TLCS. Caloxin 1b1 (100 μM) was used to inhibit PMCA activation and was added to appropriate wells 30 min prior to the addition of RNLS. After treatments, 50 μl of cell-free medium was removed to assay for amylase secretion. The remaining medium containing the pancreatic lobules was homogenized by hand using 10 strokes in a 2-ml glass Teflon homogenizer. The homogenate was centrifuged at 500 × g for 10 min, and the resulting supernatant was used to assay zymogen activation and amylase secretion.

Zymogen activation assays

The supernatant from pancreatic lobules was assayed for zymogen activity using fluorogenic substrates as described (21). Briefly, 50 μl of enzyme substrate (40 mM final) (chymotrypsin from Calbiochem; trypsin from Peptides International, Louisville, KY) was added to each well containing 20–50 μl of sample and 400–430 μl of assay buffer (50 mM Tris (pH 8.1), 150 mM NaCl, and 1 mM CaCl2) for a total volume in the well of 500 μl. The plate was then read using a fluorometric plate reader (Flx800, BioTek Instruments, Winooski, VT) at an excitation wavelength of 380 nm and emission wavelength of 440 nm for...
In vivo model of pancreatitis and renalase post-treatment

Mice (∼25 g) were fasted overnight and then given six hourly i.p. injections of cerulein (50 μg/kg of body weight in 200 μl of PBS) to induce pancreatitis. For studies that examined the effects of renalase administration postinduction of pancreatitis, rRNLS (4 mg/kg of body weight in 200 μl of PBS) was given concurrently with the third CER injection. Tissues and blood were collected for assay at relevant time points (all time points are designated as time after first CER injection).

Histology and immunostaining

Pancreatic lobules (in vitro) or tissue pieces (in vivo) were placed into cassettes and fixed in normal buffered formalin. Specimens were washed into 70% ethanol and brought to the Yale Pathology Tissue Services Lab for paraffin embedding, sectioning, and processing for H&E and immunostaining. Histology slides were examined by light microscopy at 400×, and either histologic markers were scored or immunoreactive cells were counted. Histologic scoring was done based on previously described criteria (22). All histology images were photographed at 400×.

Immunofluorescence

Tissues were removed, cut into small (about 10-mm) cubes, and fixed in 2% paraformaldehyde in PBS for 30 min on ice. Tissues were then washed and placed into 15% sucrose in PBS overnight at 4 °C. The next day tissues were placed into Cryomolds (Sakura Finetek, Torrance, CA) and snap frozen on dry ice in O.C.T. Compound (Sakura Finetek). Thin sections were molds (Sakura Finetek, Torrance, CA) and snap frozen on dry ice in O.C.T. Compound (Sakura Finetek). Thin sections were then rinsed in IF buffer twice at room temperature. Primary antibody anti-PMCA4b (JA-3, Santa Cruz Biotechnology, Dallas, TX) was added to slides at a dilution of 1:100 in IF buffer and incubated overnight at 4 °C in a humidified chamber. The next day slides were washed twice with IF buffer with 0.2% BSA for 10 min each followed by washing twice in IF buffer for 10 min each. Second antibody was then added (Alexa Fluor 488 at 1:500 dilution) in IF buffer and incubated at room temperature for 1 h. Thirty minutes into the second antibody incubation phallolidin conjugated to Alexa Fluor 555 was added to slides to double label for F-actin during the remaining 30 min. Slides were then washed twice with IF buffer with 0.2% BSA for 5 min each followed by washing twice with IF buffer for 5 min each and finally once with TBS (pH 7.5) for 5 min. Lastly, slides were mounted with VECTASHIELD hard set mounting medium with DAPI (Vector Labs, Burlingame, CA). When dry, slides were examined using a fluorescence microscope (Zeiss Axio-phot), and images were captured using a digital camera (Spot Imaging).

Amylase assay

Samples were thawed on ice, and the cell-free supernatant was assayed for secreted amylase. The remaining sample (lobule plus medium) from the zymogen activation assays was assayed for total amylase. Amylase activity was determined using a commercial kit. Amylase secretion was calculated as percent total release (medium/medium + cells). Plasma amylase content was measured according to the manufacturer’s instructions (Phaebadas kit, Magle Life Sciences, Lund, Sweden).

PCR

All PCR primers are described in Table 1. The mouse primers for PMCA1–4 (23, 24) and PMCA4a and PMCA4a/b (25) have been described. The human PMCA primers were designed using Primer-BLAST (National Institutes of Health). Human cDNA was provided by Guy Groblewski of the University of Wisconsin. Mouse cDNA was produced from total RNA isolated from mouse pancreatic acini, pancreas, and kidney using TRIzol (Ambion/Thermo Fisher, Grand Island, NY). First-strand cDNA was prepared using a Verso cDNA synthesis kit (Thermo Fisher) following the manufacturer’s protocol. PCR was carried out using a Platinum Taq DNA polymerase kit (Invitrogen) and 1 μl of first-strand cDNA in a 50-μl reaction volume containing 1× PCR Buffer (from the kit), 1.65 mM MgCl₂, 200 mM each deoxynucleotide triphosphate, 1.25 units of Taq DNA polymerase (Invitrogen), and 200 nm each primer (Table 1). For amplification of PMCA isoforms, the following conditions were used: initial denaturation for 2 min at 95 °C and then 40 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 30 s). This was followed by a single extension step for 1 min at 72 °C. PCR products were analyzed on 1% agarose gels with ethidium bromide.

MTT assay

The reduction of MTT to formazan is a measure of redox activity and has been used as a marker of cell viability as well as cell proliferation. It is generally thought that this reaction takes place in mitochondria, but a recent study has shown only about 25% of MTT reduction is associated with mitochondria; the remainder takes place at a distance by non-mitochondrial enzymes. This suggests that MTT reduction can be used as a more general marker of redox activity (26). Here, we used MTT reduction to formazan as a general marker of metabolic health. To assay MTT activity, a stock MTT solution (5 mg/ml) was made by dissolving MTT (Invitrogen) in sterile Dulbecco’s PBS. The solution was filtered using a 0.2-μm filter unit and stored at −20 °C protected from light. Pancreatic lobules were isolated and treated as indicated above. After 30 min of CER treatment, 100 μl of stock MTT was added to each well (0.45 mg/ml final) for 1 h. Lobules were removed from the wells, blotted dry, and homogenized in 750 μl of buffer (40% dimethylformamide, 2% glacial acetic acid, 16% sodium dodecyl sulfate, and 42% water (pH 4.7)) using a conical tip homogenizer. The homogenate was incubated at 37 °C for 2 h. The homogenate was then centrifuged at 10,000 × g for 5 min, and the resulting supernatant was read at 520 nm. Values were normalized to DNA (A₂₆₀ nm) and activity was expressed as a ratio of MTT (520 nm)/DNA (A₂₆₀ nm).
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**Table 1**

| Gene     | GenBankTM accession number | Primer sequence | Expected size | Refs. |
|----------|----------------------------|-----------------|---------------|-------|
| **Mouse** |                            |                 |               |       |
| PMCA1    | NM_026482                  | F, 5'-TCT GGC TAC GGA ACC ACC CA-3' | 390           | 4, 9  |
|          |                            | R, 5'-AAA GGC TTC CCG CCA AAC TG-3' |               |       |
| PMCA2    | NM_009723                  | F, 5'-CAC CCT GCT CTT GTG GGG TG-3' | 355           | 4, 9  |
|          |                            | R, 5'-AGG GAT GGT GGC GAT GAC CT-3' |               |       |
| PMCA3    | NM_177236                  | F, 5'-ATG AAG AGC TGG CCG CAG AGG GG-3' | 345           | 4, 9  |
|          |                            | R, 5'-GGG TGG TGG GTA AGA TGC CG-3' |               |       |
| PMCA4    | NM_213616                  | F, 5'-CAA GCC TCT GAT CTC CCC CA-3' | 364           | 4, 9  |
|          |                            | R, 5'-CCA CTG CTC CAT GGT CAG GC-3' |               |       |
| PMCA4a   | NM_001167949               | F, 5'-GCA CTG GAT GTG GTG TCT CTT TAT-3' | 417           | 11    |
|          |                            | R, 5'-CAG TCA CAG CCC ACA ACA GGA GCA-3' |               |       |
| PMCA4a/b | (a) NM_001167949/(b) NM_213616.3 | F, 5'-GGA CGA GAT TGA CTC CCG CG-3' | (a) 469/b 278 | 11    |
|          |                            | R, 5'-CAC CAT CCA ACA ACA GGA GCA CAC T-3' |               |       |
| **Human**|                            |                 |               |       |
| PMCA1    | NM_001001323.1             | F, 5'-AGC ACT GTG TCA GAC CAA CG-3' | 555           | a     |
|          |                            | R, 5'-AGG AGC ATG CAA AGG AGG AT-3' |               |       |
| PMCA2    | NM_001001331.2             | F, 5'-GAA ACC GTG CAG GAT TGG TG-3' | 179           | a     |
|          |                            | R, 5'-GTG AGC TCG AAC AGG TGG CC-3' |               |       |
| PMCA3    | NM_021949.3                | F, 5'-GCT CAG TGT CTC AGT TGT TTG TG-3' | 157           | a     |
|          |                            | R, 5'-GAT CTC TCG CTC GCC TTC TTG GG-3' |               |       |
| PMCA4a/b | (a) NM_001001396.2/(b) NM_001684.4 | F, 5'-CCG CAA TAC CTA CCC GAT CC-3' | (a) 597/b 419 | 19    |
|          |                            | R, 5'-GCA ATG CTC CAG CGT ATT GTT TTG-3' |               |       |

*Primer design using Primer-BLAST.

**Measurement of edema**

Pancreatic tissue was harvested, and its wet weight was determined. Tissues were then dried at 60 °C for at least 48 h and reweighed. Edema was expressed as percent wet weight (wet weight − dry weight/wet weight × 100).

**Statistical analysis**

Data represent the mean ± S.E. of at least three individual experiments unless otherwise noted with each performed in at least duplicate. Student’s t test analysis was used to determine statistical significance for in vitro experiments, whereas a Mann–Whitney test was used for in vivo experiments. A p value of <0.05 was assigned significance.

**Author contributions**—T. R. K. designed and performed experiments (Figs. 1, 2, and 4–9), analyzed data, prepared figures, and wrote the initial draft of the manuscript. A. M. R. designed and performed experiments (Figs. 1, 4, 5, and 7), analyzed data, and prepared figures. K. D. performed experiments (Fig. 6) and analyzed data. C. S. designed and performed experiments (Fig. 3), analyzed data, and prepared figures. V. P. performed experiments (Figs. 1 and 7–9). S. -L. C. performed experiments (Figs. 1, 7, and 8). G. V. D. designed and produced the renalase knock-out mouse, discussed results, and revised the manuscript. F. S. G. designed experiments, discussed results, and revised the manuscript.

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