Stanniocalcin-1 Inhibits Renal Ischemia/Reperfusion Injury via an AMP-Activated Protein Kinase-Dependent Pathway

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ABSTRACT
AKI is associated with increased morbidity, mortality, and cost of care, and therapeutic options remain limited. Reactive oxygen species are critical for the genesis of ischemic AKI. Stanniocalcin-1 (STC1) suppresses superoxide generation through induction of uncoupling proteins (UCPs), and transgenic overexpression of STC1 inhibits reactive oxygen species and protects from ischemia/reperfusion (I/R) kidney injury. Our observations revealed high AMP-activated protein kinase (AMPK) activity in STC1 transgenic kidneys relative to wild-type (WT) kidneys; thus, we hypothesized that STC1 protects from I/R kidney injury through activation of AMPK. Baseline activity of AMPK in the kidney correlated with the expression of STCs, such that the highest activity was observed in STC1 transgenic mice followed (in decreasing order) by WT, STC1 knockout, and STC1/STC2 double-knockout mice. I/R in WT kidneys increased AMPK activity and the expression of STC1, UCP2, and sirtuin 3. Inhibition of AMPK by administration of compound C before I/R abolished the activation of AMPK, diminished the expression of UCP2 and sirtuin 3, and aggravated kidney injury but did not affect STC1 expression. Treatment of cultured HEK cells with recombinant STC1 activated AMPK and increased the expression of UCP2 and sirtuin 3, and concomitant treatment with compound C abolished these responses. STC1 knockout mice displayed high susceptibility to I/R, whereas pretreatment of STC1 transgenic mice with compound C restored the susceptibility to I/R kidney injury. These data suggest that STC1 is important for activation of AMPK in the kidney, which mediates STC1-induced expression of UCP2 and sirtuin 3 and protection from I/R.


AKI is common and frequently associated with high morbidity and mortality. A study by Chertow et al.1 showed that a small increase in serum creatinine in patients admitted to the hospital is associated with higher mortality, longer hospitalization, and higher cost of care. Ischemic kidney injury may not recover and could lead to end stage kidney failure, or it may exacerbate other underlying disease processes and accelerate the progression to end stage kidney failure.2 For example, a short ischemic episode in the mouse leads to persistent interstitial fibrosis,3 whereas in humans, acute ischemic insult occurring immediately after kidney transplantation may delay graft function and lead to chronic allograft nephropathy.4,5 Experimental mouse
ischemia/reperfusion (I/R) kidney injury has been used extensively to study the pathogenesis of ischemic AKI. Increased generation of reactive oxygen species (ROS), endothelial dysfunction, vascular congestion at the corticomedullary junction, and inflammation are critical players in the pathogenesis of I/R kidney injury.6–10 Although the pathogenesis of ischemic kidney injury is better defined, few therapeutic options are currently available.

Mammalian stanniocalcin-1 (STC1) is ubiquitously expressed, and the highest level of expression is in the ovary, kidney, prostate, and thyroid.11 STC1 is an intracellular-acting, extracellular signaling protein (paracrine/intracrine)12; it binds to a cell-surface protein followed by internalization and targeting to the inner mitochondrial membrane.13 Mammalian STC2 is also ubiquitously expressed; cumulative evidence suggests that it also acts in an autocrine/paracrine manner and that its functions are linked to the endoplasmic reticulum.14 STC1 suppresses superoxide generation through induction of uncoupling proteins (UCPs),15,16 stabilizes endothelial barrier function,17 and diminishes macrophage mobility and response to chemoattractants.18 Thus, STC1 may target core pathways in the pathogenesis of I/R kidney injury. Indeed, we have recently shown that transgenic (Tg) overexpression of STC1 protects from I/R kidney injury in mice through inhibition of ROS and inflammation.16 Current observations reveal increased activity of AMP-activated protein kinase (AMPK) in STC1 Tg kidneys. AMPK regulates cellular metabolism and is a key component of the cellular adaptive responses to ischemia in the kidney.19,20 Recent studies suggest that pretreatment with AMPK activators (5-aminoimidazol-4-carboxamide-1-β-d-ribofuransoide and metformin) may have a protective role in renal I/R injury.21 We, therefore, hypothesized that STC1 protects from I/R through activation of AMPK.

Our findings in these experiments are consistent with this hypothesis: baseline activity of AMPK in the kidney correlates with the expression of STC1 and STC2, with the highest activity observed in STC1 Tg followed (in decreasing order) by wild type (WT), STC1 knockout (KO), and STC1/STC2 double KO. I/R in WT kidneys increases AMPK activity and the expression of STC1, UCP2, and silent mating type information regulation 2 homolog 3 (SIRT3; also known as sirtuin 3). UCPs limit free radical production,22,23 whereas SIRT3, a mitochondrial acet-tyltransferase, decreases ROS through a number of pathways,24 but its functions in the kidney are largely unknown. Pretreatment of WT mice with 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (compound C [CC]), a pharmacologic inhibitor of AMPK,25,26 abolishes I/R-induced AMPK activation, diminishes the expression of SIRT3 and UCP2, and is associated with increased ROS production and worse morphologic changes. In agree-ment with these data, treatment of human embryonic kidney (HEK) cells with recombinant STC1 (rSTC1) activates AMPK and increases the expression of SIRT3 and UCP2, whereas concomitant treatment with CC abolishes the responses to rSTC1. These data are consistent with placement of STC1 upstream of AMPK, whereas UCP2 and SIRT3 appear to be downstream of AMPK. Importantly, STC1 KO mice display greater susceptibility to I/R kidney injury compared with WT mice, and pretreatment of STC1 Tg mice with CC restored susceptibility to I/R kidney injury. The data suggest that (1) STC1 is important for AMPK activation in the kidney and (2) STC1-induced protection from I/R and expression of UCP2 and SIRT3 are AMPK-dependent.

RESULTS

STC1 Regulates AMPK Activity in the Kidney
To determine whether AMPK activity in the kidney correlates with the expression of STC1, we compared whole-kidney lysates from WT and STC1 Tg mice and found higher-level activity of AMPK in STC1 Tg kidneys compared with WT kidneys (Figure 1A). Quantitatively, STC1 Tg kidneys displayed 2.5-fold higher baseline activity of AMPK relative to WT (Figure 1A). Staining for phosho-AMPK (pAMPK) revealed the highest activity of AMPK in STC1 Tg kidneys followed (in decreasing order) by WT, STC1 KO,28 and STC1/STC2 double-KO29 kidneys (Figure 1B). Staining for pAMPK is observed in the entire kidney, including tubules, glomeruli, and arterioles; tubular staining appears diffusely cytoplasmic, with some tubule segments showing stronger staining than others. Our data suggest that STC1 is an important regulator of AMPK in the kidney. Using antibodies that recognize both α1 and α2 isoforms of the catalytic subunits of AMPK, we observe the phosphorylated α1 (larger-sized band) consistently and phosphorylation of the α2 (smaller-sized band) intermittently in WT mice; however, in STC1 Tg mice, we detect phosphorylation of both isoforms consistently (albeit at varying intensity) (Figure 1A). STC1 shows preferential activation of the α2 isofrom; however, the significance of these observations remains to be determined.

Inhibition of AMPK Exacerbates I/R Kidney Injury
Tg overexpression of STC1 confers resistance to I/R kidney injury through suppression of oxidant stress and inflammation,16 whereas acute knockdown of STC1 in the kidney leads to severe proximal tubule injury and kidney failure.30 AMPK regulates cellular metabolism, and recent studies suggest that pretreatment with AMPK activators (5-aminoimidazol-4-carboxamide-1-β-d-ribofuransoide and metformin) may protect from I/R injury.20,21,31 To determine whether AMPK is important for protection from I/R kidney injury and test whether STC1 acts upstream of AMPK, we pretreated WT mice with CC before I/R. Pretreatment with CC decreased I/R-induced activation of AMPK by one half, compared with responses in vehicle-pretreated mice (Figure 2). Seventy-two hours after I/R, we observed increased cellular vacuolization and tubular dilation, predominantly in the cortex (Figure 3A), coupled with significant elevation in both H2O2 and superoxide in CC-pretreated mice compared with vehicle-treated WT controls (Figure 3B) but no significant differences in serum creatinine, creatinine clearance (CrCl), or urine output (Figure 4). The apparent discrepancy
between the severe morphologic changes observed at the 72-hour time point but no apparent difference in CrCl in CC- and I/R-treated WT mice compared with vehicle- and I/R-treated WT mice may be explained by the fact that urine collection for CrCl began 48 hours after clamping (i.e., 24 hours before tissue harvest for histology) and before the full injury had been established in CC- and I/R-treated mice. Treatment of sham-operated WT mice with CC did not increase H2O2 or superoxide (Figure 3B) and did not lead to morphologic or functional changes in the kidney (Figures 3A and 4).

As shown in Figure 5, I/R induced the expression of STC1, UCP2, and SIRT3. UCPs limit free radical production, whereas SIRT3 is a mitochondrial acetyl transferase known to decrease ROS through a number of pathways. Pretreatment with CC prevented I/R-induced increase in UCP2 and SIRT3 (Figure 5, B and C), consistent with AMPK-mediated regulation of UCP2 and SIRT3. However, AMPK inhibition did not affect I/R-induced expression of STC1 protein (Figure 5A). In addition, treatment of cultured HEK cells with rSTC1 activates AMPK, and this activation precedes the increase in UCP2 and SIRT3 expression (Figure 6A), whereas concomitant treatment with CC abolishes the responses to rSTC1 (Figure 6B). These observations support the in vivo data and are consistent with STC1-induced activation of AMPK and AMPK-mediated induction of SIRT3 and UCP2, thus placing STC1 upstream of AMPK, whereas UCP2 and SIRT3 are downstream of AMPK. Taken together, these data suggest that STC1 may protect from I/R kidney injury through activation of AMPK to increase the expression of UCP2 and SIRT3 and decrease ROS production.

Inhibition of AMPK in STC1 Tg Mice Restores Susceptibility to I/R Kidney Injury

To show that activation of AMPK mediates the renoprotective effects that we observed using anti-pAMPKα1,α2. Bar graph represents quantification of pAMPKα1,α2 staining from four mice for each group, and data are presented as mean ± SEM. A, arterioles; G, glomeruli; T, tubules; V, venules.
In STC1 Tg mice, we pretreated these mice with CC or vehicle before I/R. Pretreatment of STC1 Tg mice with CC diminished AMPK activity (Figure 7A) and resulted in severe kidney injury after I/R, which was characterized by cellular vacuolization, tubular dilation, and cast formation (predominantly in the cortex) (Figure 7B), similar to those changes observed in WT kidneys after I/R, increased serum creatinine, decreased CrCl and urine output (Figure 7C), and increased superoxide and H2O2 generation (Figure 7D). In addition, pretreatment with CC attenuated the expression of UCP2 and SIRT3 in STC1 Tg kidneys (Figure 8). However, pretreatment of STC1 Tg mice with vehicle did not produce any of these changes. The data suggest that, in STC1 Tg mice, protection from I/R, induction of UCP2 and SIRT3, and suppression of ROS are all AMPK-mediated.

**STC1 KO Mice Display Increased Susceptibility to I/R**

To lend additional support for the above data, we carried out I/R experiments in STC1 KO mice, an alternate model of low AMPK activity (Figure 9). Of note, baseline expression of

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**Figure 2.** Treatment with CC blocks I/R injury-induced activation of AMPK in WT mice. (A) Immunohistochemistry of kidney sections using anti-pAMPKα1,α2. Representative images from two mice per group are shown. (B) Bar graph represents quantification of pAMPKα1,α2 staining. Data represent the mean±SEM from four mice for each group. (C) Total kidney lysates from vehicle sham, CC sham, vehicle I/R, or CC I/R mice were resolved on SDS-PAGE, and Western blots were reacted with anti-pAMPKα1,α2, anti-AMPKα1,α2, and anti-actin; representative Western blots are shown. Bar graphs depict the ratio of pAMPKα1,α2/total AMPKα1,α2, pAMPKα1,α2/actin, and total AMPKα1,α2/actin. Data represent the mean±SEM from four mice for each group. tAMPK, total AMPK; Veh, vehicle. *P<0.05 versus vehicle sham.
UCP2 and SIRT3 is low in STC1 KO kidneys, and we observe no change in SIRT3 and UCP2 levels after I/R (Figure 9), consistent with STC1-induced and AMPK-mediated expression of both SIRT3 and UCP2. Of interest, histology revealed mild kidney injury in sham-treated STC1 KO mice compared with sham-treated WT mice. The data suggest that, absent STC1 expression and hence, diminished AMPK activity at baseline, the kidneys manifest injury after the stress of sham operation; these observations are consistent with recent findings from our laboratory, where acute and kidney-specific knockdown of STC1 leads to AKI. I/R produced greater kidney injury in STC1 KO mice compared with WT mice (Figure 10). This injury was characterized by higher serum creatinine, lower CrCl (Figure 11), greater ROS generation (Figure 12), diffuse cellular vacuolization, and marked tubular dilation as well as cast formation that involved approximately 30% of the tubules in the cortex (Figure 10). Morphologically, the injury appeared worse than that observed in WT or STC1 Tg mice after CC treatment and I/R. These data suggest that STC1 expression and pre-activation of AMPK before I/R are critical for the protection of the kidney from I/R (see Discussion).

SIRT3 Induces UCP2 in the Kidney and Decreases Superoxide Generation

The mitochondria account for the majority of cellular ROS production. Recent pub-

Figure 3. Inhibition of AMPK exacerbates I/R kidney injury in WT mice. (A, a) Periodic acid-Schiff staining of kidney sections for morphology showed severe cellular vacuolization (predominantly in the cortex) 72 hours after I/R in WT mice pretreated with CC. Representative images from two mice per group are shown. (A, b) Bar graph represents quantification of tubular injury and depicts the mean±SEM of the percentage of tubules with cellular vacuolization, dilation, and/or cast formation (n=4 mice/group). (B) AMPK inhibition in WT mice subjected to I/R kidney injury increases H$_2$O$_2$ and superoxide production. (B, a) WT mice were treated with CC or vehicle before I/R or sham surgery, and superoxide (MitoSOX red fluorescence) was measured in tubular epithelial cells 72 hours after I/R or sham surgery. Representative images from two mice per group are shown; blue fluorescence corresponds to 4’,6-diamidino-2-phenylindole. Bar graphs show (B, b) H$_2$O$_2$ and (B, c) superoxide 72 hours after I/R or sham surgery; data represent mean±SEM from four mice per group. Veh, vehicle. *P<0.05 versus vehicle sham.
lished works suggest that mitochondrial SIRT3 activates several enzymes that are critical for the regulation of cellular ROS.24,32,33 In brown adipose tissue, increased expression of SIRT3 upregulates the expression of UCP1.34 Because SIRT3 expression is induced by I/R and attenuated by pretreatment with CC (Figure 5), we hypothesized that SIRT3 is involved in the regulation of UCP2 and superoxide in the kidneys. Indeed, SIRT3 Tg kidneys express higher baseline levels of UCP2 relative to WT, which correlates with lower baseline superoxide generation (Figure 13), similar to observations that we made in STC1 Tg kidneys.16 As observed in WT mice, where pretreatment with CC attenuated I/R-induced UCP2 and SIRT3 expression, pretreatment of STC1 Tg mice with CC decreases the expression of UCP2 and SIRT3 at both baseline and post-I/R (Figure 8). Similarly, in cultured HEK cells, treatment with CC abolishes STC1-induced expression of UCP2 and SIRT3 (Figure 6B). Collectively, our data suggest that STC1 confers resistance to I/R kidney injury through activation of AMPK, leading to induction of the mitochondrial proteins SIRT3 and UCP2 and suppression of superoxide generation.

**DISCUSSION**

Our findings identify STC1 as a regulator of AMPK, UCP2, and SIRT3 in the kidney and bring new insight into the role of STC1 in kidney physiology. Baseline activity of AMPK in the kidney STC1 KO mice, an alternate model of low AMPK activity, display greater susceptibility to I/R kidney injury. Thus, activation of AMPK in the kidney seems to be critical for STC1-induced suppression of superoxide and cytoprotection, and the protective effects of STC1/AMPK are possibly mediated through UCP2 and SIRT3.

STC1 is a stress gene; it responds to various stimuli, including hypoxia35 and cytokines.36 The expression of STC1 after I/R is likely a response to the resulting hypoxia and/or release of cytokines; however, previous work showed that stress-induced expression of STC1 may lag considerably,36 as does the expression of UCPs in response to STC1.15 Thus, effective protection by STC1 requires pre-exposure of the cells to STC1 or preactivation of AMPK.21 Indeed, Tg overexpression of STC1 (pre-exposure) inhibits ROS and renal I/R injury in mice16; AMPK activity is elevated in STC1 Tg mice at baseline (relative to WT) and does not change after I/R (Figure 7A). In contrast, STC1 KO mice are more susceptible to I/R kidney injury (relative to WT); consistently, AMPK activity is low in STC1 KO mice at baseline and does not change after I/R (Figure 9). Thus, the timing of STC1 expression and/or AMPK activation before I/R is important for kidney protection rather than the level of AMPK activity in response to stress or injury.

The metabolic sensor AMPK regulates energy-generating and -consuming pathways; it is highly expressed in the kidney and involved in kidney physiology.20

**Figure 4.** AMPK inhibition in WT mice exacerbates kidney injury post-I/R. (A) Serum creatinine, (B) CrCl normalized to weight, and (C) timed urine output were carried out as detailed in Concise Methods. Bar graphs depict the mean±SEM (n=9 mice for WT baseline; n=3 mice for CC sham; n=4 mice/group for Veh sham, Veh I/R, or CC I/R). UOP, urine output; Veh, vehicle. *P<0.05 versus WT baseline.
Sodium transport consumes large amounts of energy, and AMPK couples ion transport to cellular energy metabolism. AMPK is activated in response to high dietary salt; it regulates several ion transporters, including the Na⁺,K⁺-ATPase, the epithelial sodium channel, the Na⁺,K⁺2Cl⁻ cotransporter, and the vacuolar hydrogen pump H⁺,ATPase. AMPK has also been reported to play a role in the regulation of podocyte function and the pathogenesis of diabetic nephropathy. Activation of AMPK by adiponectin in podocytes reduces albuminuria, whereas reduced AMPK activity in the diabetic kidney is associated with accumulation of triglycerides and glycogen and results in kidney hypertrophy. Kidney ischemia activates AMPK, and cumulative data suggest that preactivation of AMPK protects from I/R kidney injury. Here, we identify STC1 as an important activator and regulator of AMPK in the kidney; by extrapolation, our data suggest an important role for STC1 in the control of transport processes in the kidney from Cl⁻ to Na⁺ and H⁺ as well as the regulation of podocyte function and diabetes.

The observation that STC1 induces SIRT3 highlights the importance of STC1 even further. Null mutations of silent information regulator 2 shorten, whereas an extra copy of silent information regulator 2 extends lifespan in yeast by 50%. The mammalian family of sirtuins consists of seven members that catalyze an NAD⁺-dependent ADP ribosylation or acetyl transfer from lysine moieties on proteins to inhibit or activate proteins, respectively. Sirtuins localize to the nucleus (SIRT1, SIRT6, and SIRT7), mitochondria (SIRT3, SIRT4, and SIRT5), and cytoplasm (SIRT2). STC1 localizes to the inner mitochondrial membrane; SIRT3 also localizes to the inner mitochondrial membrane, induces UCPs, and decreases superoxide generation in adipocytes. We, therefore, hypothesized that UCP2 expression in the kidney is SIRT3-dependent. Indeed, our observations reveal increased expression of UCP2 concomitant with lower-level superoxide (red mitochondrial superoxide indicator [MitoSOX] fluorescence) in SIRT3 Tg kidneys. Moreover, STC1 and AMPK regulate the expression of SIRT3 in the kidney. It is, therefore, possible that the cytoprotective effects of STC1 are mediated, in part, through changes in SIRT3-induced responses that may not be limited to upregulation of UCP2 and reduction in mitochondrial superoxide. Thus, our data pave the way for the discovery of novel mitochondrial pathways for cytoprotection.

In summary, our data suggest that (1) STC1 is important for AMPK activation in the kidney and that (2) STC1-induced protection from I/R and expression of UCP2 and SIRT3 are AMPK-dependent.

**CONCISE METHODS**

**Materials**

rSTC1 was purchased from MyBioSource (San Diego, CA). Goat anti-ATP5B subunit of F1-ATPase, goat anti-UCP2, and goat anti-STC1 were purchased from MyBioSource (San Diego, CA). 

**Figure 5.** Inhibition of AMPK in WT mice blocks I/R-induced expression of SIRT3 and UCP2. (A) Total kidney lysates were obtained 72 hours after I/R. Western blots were reacted with anti-STC1 and anti-GAPDH; bar graph shows mean±SEM of n=3 mice for vehicle sham and n=4 mice for all other groups. Kidney mitochondrial lysates were obtained 72 hours after I/R; Western blots were reacted with (B) anti-UCP2, (C) anti-SIRT3, and (B and C) anti-F1-ATPase. Bar graphs show the mean±SEM from four mice per group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Veh, vehicle. *P<0.05 versus vehicle sham.
Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-AMPKα1/α2 was purchased from Signalway (Atlanta, GA). Rabbit anti-pAMPKα1/α2 (recognizes phosphorylated Thr172) was purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-SIRT3 was purchased from EMD Millipore (Billerica, MA). Rabbit anti-actin was purchased from Sigma-Aldrich (St. Louis, MO). MitoSOX was purchased from Invitrogen (Carlsbad, CA). Creatinine measurement kit was purchased from BioAssay Systems (Hayward, CA).

Mice
The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and animal experiments were approved by university ethics review boards. STC1 Tg mice were generated by Varghese et al.27 and made available for our studies (derived from line 2). STC1 Tg is driven by the metallothionein I minimal promoter over C57B/6 genetic background,27 and their phenotype has been described in previous studies.16,27,50 SIRT3 Tg is driven by the cytomegalovirus early enhancer/chicken actin promoter on C57B/6 background; mice display no overt phenotype (unpublished data). STC1 KO and STC1/STC2 double KO are also on C57B/6 genetic background and have been described elsewhere.28,29 All studies are carried out using mice homozygous for the transgene or WT mice derived from heterozygous breeders of the respective lines. Mice are maintained in the mean±SEM of three independent experiments depicting densitometry values of pAMPKα1,α2, SIRT3, or UCP2 normalized to actin. P<0.05 versus time 0 minutes (0) for #SIRT3, ◆UCP2, and *pAMPK. (B) STC1-induced increase in SIRT3 and UCP2 expression is AMPK-dependent. On the basis of the time courses for activation of AMPK and induction of SIRT3 and UCP2 (shown in A), cultured HEK cells were treated with rSTC1 plus vehicle or rSTC1 and CC for the specified time points. Western blots were reacted with anti-pAMPKα1,α2, anti-AMPKα1,α2, anti-actin, anti-SIRT3, and anti-UCP2. The graph represents the mean±SEM of three independent experiments. tAMPK, total AMPK; Veh, vehicle.

Figure 6. STC1 increases SIRT3 and UCP2 expression in vitro via AMPK. (A) STC1 activates AMPK and increases the expression of SIRT3 and UCP2 in vitro. Cultured HEK cells were treated with rSTC1 for different time points. Western blots were reacted with anti-pAMPKα1,α2, anti-AMPKα1,α2, anti-actin, anti-SIRT3, and anti-UCP2. The graph represents the mean±SEM of three independent experiments depicting densitometry values of pAMPKα1,α2, SIRT3, or UCP2 normalized to actin. P<0.05 versus time 0 minutes (0) for #SIRT3, ◆UCP2, and *pAMPK. (B) STC1-induced increase in SIRT3 and UCP2 expression is AMPK-dependent. On the basis of the time courses for activation of AMPK and induction of SIRT3 and UCP2 (shown in A), cultured HEK cells were treated with rSTC1 plus vehicle or rSTC1 and CC for the specified time points. Western blots were reacted with anti-pAMPKα1,α2, anti-AMPKα1,α2, anti-actin, anti-SIRT3, and anti-UCP2. AMPK activity (pAMPK) was sampled 15 minutes after treatment with rSTC1, whereas the expressions of SIRT3 and UCP2 were sampled 3 hours after treatment with rSTC1. Graphs represent the mean±SEM of three independent experiments. tAMPK, total AMPK; Veh, vehicle.
air-conditioned rooms under pathogen-free conditions with 12-/12-hour light/dark cycles and given free access to food and water during the experiments.

**AKI Model**

We used an established mouse model of kidney I/R injury induced by clamping of bilateral renal pedicles. Briefly, male mice (10–25
weeks, 19–30 g; the proportion of young-to-old mice was evenly distributed between the treatment groups) are anesthetized with an intraperitoneal injection of 2 ml/kg combination anesthetic (contains 37.5 mg ketamine, 1.9 mg xylazine, and 0.37 mg acepromazine per 1 ml). After abdominal incision, left and right renal pedicles are bluntly dissected, and a nontraumatic vascular clamp (Roboz Surgical Instruments, Gaithersburg, MD) is placed on each renal pedicle for 30 minutes. During the procedure, animals are kept under heating lamp and hydrated with warm saline. After 30 minutes of ischemia, the clamps are removed, the wounds are sutured, and the animals are allowed to recover. Sham-treated animals undergo similar surgical procedures without clamping of the renal pedicles. Mice are killed at 72 hours after surgical procedure; blood samples are obtained for creatinine measurement. Kidneys are harvested for histology, immunohistochemistry, Western blotting, superoxide, and H2O2 measurements. Histologic injury was analyzed by one member of the team (I.S.-C.P.), determined based the number of tubules showing vacuolization, dilation, and cast formation, and expressed as the mean ± SEM of the percentage of tubules with cellular vacuolization, dilation, and/or cast formation.

**CC Treatment**

CC (Sigma-Aldrich) is dissolved in methanol and diluted in saline (final concentration was 1.25 mg/ml CC and 20% methanol). WT and STC1 Tg mice are given a single intraperitoneal injection of CC at 20 mg/kg or vehicle (20% methanol) 2 hours before clamping/sham surgery.

**Assessment of Renal Function**

For baseline CrCl measurement, mice are placed in metabolic cages for urine collection starting 24 hours before I/R or sham operation, and blood samples are collected for creatinine measurement before I/R or sham surgery. For CrCl measurement post-I/R or sham operation, mice are placed in metabolic cages for 24 hours of timed urine collection beginning 48 hours post-I/R or sham surgery and ending at the time of euthanasia (72 hours post-I/R); blood samples are taken at the time of euthanasia. Serum and urine creatinine are measured using the Quantichrom Creatinine Assay Kit (Jaffe method) as per the manufacturer’s instructions. CrCl is calculated and normalized to weight. Please note that this method overestimates serum creatinine (almost by a factor of two) compared with capillary electrophoresis or HPLC-based measurement of serum creatinine and thus, yields lower estimates of CrCl.

**Cell Culture**

HEK cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C under 5% CO2/95% air. For time-course experiment, cells were treated with rSTC1 (100 ng/ml) or PBS for the designated time periods, scraped, collected by centrifugation, lysed in radioimmunoprecipitation assay buffer containing phosphatase inhibitors and complete mini protease inhibitor (Roche Diagnostics, Indianapolis, IN), and stored until used. In some experiments, cells were treated with rSTC1 plus CC (2 μM in 20% methanol) or rSTC1 (100 ng/ml) plus vehicle (20% methanol) for predetermined periods followed by harvesting as above.

**Western Blotting**

Lysates representing whole kidney or kidney mitochondrial fraction from WT, STC1 Tg, or STC1 KO mice are suspended in radioimmunoprecipitation assay buffer containing phosphatase inhibitors and complete mini protease inhibitor (Roche Diagnostics), phosphatase inhibitors, trichostatin A, and nicotinamide (Sigma-Aldrich). Nicotinamide and trichostatin A are class III and class I histone deacetylase inhibitors, respectively, and they are added to prevent the deacetylation of proteins in vitro. Lysates
are centrifuged at 8000×g for 10 minutes at 4°C to remove cell debris. Equal amounts of protein are separated on 12% SDS-PAGE, transferred onto nitrocellulose membranes, and incubated overnight at 4°C with primary antibodies for pAMPK, AMPK, STC1, F1-ATPase (loading control for mitochondrial proteins), actin, SIRT3, or UCP2. After washing with PBS containing 0.1% Tween-20, the membrane is incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies are visualized using chemiluminescence. Band densities of target proteins are quantified using National Institutes of Health ImageJ Software.

AMPK Activities
Whole-kidney lysates are resolved on 12% SDS-PAGE, and Western blots are reacted with pAMPK antibodies followed by stripping and reaction with AMPK antibodies. Bands representing pAMPK are normalized to the corresponding bands representing total AMPK (pAMPK/total AMPK) or actin (pAMPK/actin) and expressed as mean±SEM.

Immunohistochemistry
Formalin- or methanol-Carnoy–fixed kidney sections (5 μm) are subjected to periodic acid–Schiff staining (for morphology), anti-pAMPK (active kinase), or anti-UCP2 followed by FITC-labeled secondary antibodies. Detection is carried out using a peroxidase enzyme-based detection system (Vector Laboratories, Burlingame, CA) or fluorescence microscopy (excitation at 488 nm). Photomicrographs were taken, and quantitation of immunostaining was carried out using NIS-elements Br 3.0 software (Nikon); two representative areas of the kidney sections (viewed at magnification of ×100 for pAMPK and ×200 for periodic acid–Schiff) were analyzed.

MitoSOX Fluorescence
Freshly isolated kidneys are sectioned coronally to obtain 1-mm-thick slices that are incubated in DMEM containing 5 μmol/L
MitoSOX reagent for 10 minutes at room temperature. MitoSOX permeates live cells and selectively targets the mitochondria. It is rapidly oxidized by superoxide and emits red fluorescence. After incubation with MitoSOX, kidney slices are rinsed in PBS, fixed in 4% paraformaldehyde overnight at 4°C, and embedded in optimal cutting temperature compound; 5-μm-thick frozen sections are stained with 4',6-diamidino-2-phenylindole and viewed under fluorescence microscope.

Figure 11. STC1 KO mice display increased susceptibility to I/R kidney injury. (A) STC1 KO or WT mice were subjected to I/R or sham surgery and serum creatinine, (B) CrCl was normalized to weight, and (C) timed urine output was carried out as detailed in Concise Methods. UOP, urine output. Bar graphs depict the mean±SEM from three mice per group. *P<0.05 versus WT baseline.

Figure 12. STC1 KO mice display increased H2O2 and superoxide levels after I/R. (A) WT or STC1 KO mice were subjected to I/R or sham surgery, and superoxide (MitoSOX red fluorescence) was measured in tubular epithelial cells 72 hours after I/R or sham surgery. Representative images from two mice per group are shown; blue fluorescence corresponds to 4',6-diamidino-2-phenylindole. (B) Bar graphs show H2O2 and superoxide 72 hours post-I/R or sham surgery; data represent mean±SEM from three mice per group. *P<0.05 versus WT sham.
**Superoxide Measurements**

Kidneys are harvested and homogenized in 250 μL sucrose buffer (composition: 0.31 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4) on ice followed by protein quantitation (Bradford method). Kidney lysates (100 μg protein/100 μL) are placed in clear flat-bottom wells (96-well plate) containing excess dihydroethidium (100 μmol/L) in a final volume of 100 μL. The absorbance (at 530 nm) is immediately measured. Absorbance values are normalized to the absorbance of wells containing equimolar concentrations of dihydroethidium alone and expressed as OD.

**H2O2 Measurements**

Kidneys are harvested and homogenized in 250 μL sucrose buffer on ice followed by protein quantitation (Bradford method). H2O2 is measured as described previously. The method takes advantage of the conversion of Fe2+ to Fe3+ in the presence of H2O2 followed by detection of Fe3+-xylenol orange complex. Briefly, kidney lysate (100 μg/20 μL) is added to 180 μL assay buffer (composition: 100 μmol/L xylenol orange, 250 μmol/L ammonium ferrous sulfate, 4 mmol/L butylatedhydroxytoluene, and 25 mmol/L H2SO4 in methanol) and incubated at room temperature for 30 minutes followed by measurement of absorbance at 560 nm. Absorbance values are normalized to the absorbance of wells containing assay buffer alone and expressed as OD.

**Isolation and Purification of the Heavy Mitochondrial Fraction from Murine Kidney**

Mitochondria are isolated from mouse kidney as previously described. Briefly, kidneys are harvested and then transferred to chilled homogenization medium (HM; composition: 0.2 M manitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, and 10 mM Hepes, pH 7.4). After rinsing, the kidney is homogenized in HM containing a protease inhibitor cocktail, 500 nM trichostatin A, and 10 mM nicotinamide. The homogenate is centrifuged on a bench-top centrifuge for 10 minutes at 1000×g (4°C). The supernatant is recovered and centrifuged for 10 minutes at 3000×g (4°C). The resulting supernatant is then aspirated, leaving a brown mitochondrial pellet. The mitochondrial pellet is then resuspended in 100 μL mitochondria lysis buffer (composition: 1% n-dodecyl-B-D-maltoside, 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, protease inhibitor cocktail, 500 mM trichostatin A, and 10 mM nicotinamide) and stored at −80°C until analyzed.

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**DISCLOSURES**

None.

**REFERENCES**


