Catalase Overexpression Prevents Nuclear Factor Erythroid 2–Related Factor 2 Stimulation of Renal Angiotensinogen Gene Expression, Hypertension, and Kidney Injury in Diabetic Mice

This study investigated the impact of catalase (Cat) overexpression in renal proximal tubule cells (RPTCs) on nuclear factor erythroid 2–related factor 2 (Nrf2) stimulation of angiotensinogen (Agt) gene expression and the development of hypertension and renal injury in diabetic Akita transgenic mice. Additionally, adult male mice were treated with the Nrf2 activator oltipraz with or without the inhibitor trigonelline. Rat RPTCs, stably transfected with plasmid containing either rat Agt or Nrf2 gene promoter, were also studied. Cat overexpression normalized systolic BP, attenuated renal injury, and inhibited RPTC Nrf2, Agt, and heme oxygenase-1 (HO-1) gene expression in Akita Cat transgenic mice compared with Akita mice. In vitro, high glucose level, hydrogen peroxide, and oltipraz stimulated Nrf2 and Agt gene expression; these changes were blocked by trigonelline, small interfering RNAs of Nrf2, antioxidants, or pharmacological inhibitors of nuclear factor-κB and p38 mitogen-activated protein kinase. The deletion of Nrf2-responsive elements in the rat Agt gene promoter abolished the stimulatory effect of oltipraz. Oltipraz administration also augmented Agt, HO-1, and Nrf2 gene expression in mouse RPTCs and was reversed by trigonelline. These data identify a novel mechanism, Nrf2-mediated stimulation of intrarenal Agt gene expression and activation of the renin-angiotensin system, by which hyperglycemia induces hypertension and renal injury in diabetic mice.

Hyperglycemia, oxidative stress, and dysregulation of the renin-angiotensin system (RAS) have long been implicated in the development and progression of diabetic nephropathy. However, the underlying molecular mechanisms remain incompletely understood. In addition to the systemic RAS, the existence of a local intrarenal RAS in renal proximal tubule cells (RPTCs) has been well documented (1). Several lines of evidence indicate that enhanced generation of reactive oxygen species (ROS) is central to the development of hypertension and RPTC apoptosis in diabetes. ROS mediate high-glucose (HG) stimulation of angiotensinogen (Agt; the sole precursor of all angiotensins) gene expression in RPTCs in vitro (2–5). Transgenic (Tg) mice specifically overexpressing rat (r) Agt (rAgt) in their RPTCs has been well documented (1). Several lines of evidence indicate that enhanced generation of reactive oxygen species (ROS) is central to the development of hypertension and RPTC apoptosis in diabetes. ROS mediate high-glucose (HG) stimulation of angiotensinogen (Agt; the sole precursor of all angiotensins) gene expression in RPTCs in vitro (2–5). Transgenic (Tg) mice specifically overexpressing rat (r) Agt (rAgt) in their RPTCs develop hypertension and kidney injury (6). Hyperglycemia and Agt overexpression act in concert to induce hypertension and RPTC apoptosis in diabetic Agt-Tg mice (7,8). Conversely, apocynin treatment (9) and catalase (Cat) overexpression attenuate
hypothesis and RPTC apoptosis in non-diabetic Agt/Cat-Tg (10) and diabetic Cat-Tg mice (11–13).

Nuclear factor erythroid 2–related factor 2 (Nrf2) functions as a master regulator of redox balance in cellular cytoprotective responses (14). Nrf2 is normally sequestered in the cytoplasm by a cytosolic repressor, Keap1 (Kelch-like ECH-associated protein 1) and is constantly degraded (15). However, with oxidative stress Nrf2 is released from Keap1 repression, translocates to the nucleus, forms heterodimers with small musculoaponeurotic fibrosarcoma proteins, binds to antioxidant response elements, and initiates the transcription of an array of genes (16). Little information is available about the functional relationship between ROS and Nrf2 and Agt gene expression in diabetic RPTCs, which may be critical for the development of diabetic renal injury.

In the current study, we investigated the relationship between oxidative stress, Nrf2 and Agt gene expression, hypertension development, and RPTC injury in the HG milieu both in vivo and in vitro. We report that Cat overexpression prevented hyperglycemia-induced stimulation of Nrf2, heme oxygenase-1 (HO-1), and Agt gene expression in RPTCs, subsequently attenuating hypertension and ameliorating renal injury in diabetic Akita mice. In vitro, HG, hydrogen peroxide (H2O2), and the Nrf2 activator oltipraz stimulated Nrf2, HO-1, and Agt gene expression in RPTCs, which can be reversed by trigonelline (a Nrf2 inhibitor), small interfering RNAs (siRNAs) of Nrf2, antioxidants, and pharmacological blockade of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor-κB (NF-κB) signaling. Consistently, in vivo administration of oltipraz stimulated Nrf2, HO-1, and Agt gene expression in mouse renal proximal tubules (RPTs), which was reversed by trignonelline coadministration.

RESEARCH DESIGN AND METHODS

Chemicals and Constructs

D-Glucose, D-mannitol, H2O2, oltipraz (a Nrf2 activator), the alkaloid trigonelline (C7H7NO2, a Nrf2 inhibitor), PD98059 (a p44/42 MAPK inhibitor), wortmannin (an inhibitor of phosphatidylinositol 3-kinase), and anti-β-actin monoclonal antibody were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). SB203580 (an inhibitor of p38 MAPK) was obtained from Cell Signaling Technology (distributed by New England Biolabs, Whitby, Ontario, Canada). Pyrrolidine dithiocarbamate ammonium (PDTC) and Bay 11-7082 (inhibitors of NF-κB activation) were from Calbiochem (San Diego, CA). Normal glucose (5 mmol/L) glucose, DMEM (catalog no. 12320), penicillin/streptomycin, and FBS were procured from Invitrogen (Burlington, Ontario, Canada). Anti-Nrf2 and anti-Keap1 antibodies were obtained from BD Biosciences (Mississauga, Ontario, Canada) and R&D Systems (Minneapolis, MN), respectively. Polyclonal anti-HO-1 antibodies were purchased from Assay Designs (Ann Arbor, MI). Rabbit polyclonal antibodies specific for rAgt (17) were generated in our laboratory (J.S.D.C.). The plasmid pKAP2 containing the kidney-specific androgen-regulated protein (KAP) promoter that is responsive to androgen was a gift from Dr. Curt D. Sigmund (University of Iowa, Iowa City, IA) (18). The plasmid pcDNA3.1 containing the Flag-(Rel A) p65 cDNA was a gift from Dr. Marc Servant (Faculty of Pharmacy, Université de Montréal, Montréal, Québec, Canada). Full-length rCat cDNA fused with HA-tag (which encodes amino acid residues 98–106 of human influenza virus hemagglutinin at the carboxyl terminus with the Nor1 site at both the 5’ and 3’ termini) was inserted into plasmid pKAP2 at the Norl site (11). The rAgt gene promoter (N-1495 to N+18) (19) and the rat Nrf2 gene promoter (N-1980 to N+111) (20) were cloned from respective rat genomic DNA by conventional PCR with specific primers and inserted into pGL4.20 vector via KpnI and HindIII restriction sites (19). QuikChange Site-Directed Mutagenesis Kits from Stratagene (La Jolla, CA) were used to delete putative Nrf2-restriction enzymes (REs) in the rAgt gene promoter (21). Supplementary Table 1 details the oligo primers for cloning of the rAgt and rNrf2 gene promoters and site-directed mutagenesis. Scrambled Silencer Negative Control #1 siRNA and Nrf2 siRNA were obtained from Ambion (Austin, TX). Oligonucleotides were synthesized by Invitrogen. REs and modifying enzymes were obtained from commercial sources. Viable and fertile mice heterozygous for the Akita spontaneous mutation of insulin 2 (Ins2) gene (C57BL/6-Ins2Akita/J) were purchased from The Jackson Laboratory (Bar Harbor, ME; http://jaxmice.jax.org).

Generation of Akita Cat-Tg Mice

We generated Tg mice that specifically overexpress Cat in their RPTCs by cross-breeding homozygous Cat-Tg mice (11) with heterozygous Akita mice (note: homozygous Akita mice are infertile). These mice are homozygous for the Cat transgene but heterozygous for the Ins2 gene mutation (8,13).

Pathophysiological Studies

Male adult (16-week-old) non-Akita wild-type (WT), Cat-Tg, Akita, and Akita Cat-Tg mice (eight per group) were used. All animals received standard mouse chow and water ad libitum. Animal care and experimental procedures were approved by the Animal Care Committee at the Research Centre, Centre Hospitalier de l’Université de Montréal.

Systolic blood pressure (SBP) was monitored with a BP-2000 tail-cuff pressure machine (Visitech Systems, Apex, NC) in the morning, at least two to three times a week, for 5 weeks (6–13,19).

The glomerular filtration rate (GFR) was estimated according to the protocol described by Qi et al. (22), as recommended by the Animal Models of Diabetic Complications Consortium (http://www.diacomp.org/) (13,19).

Body weight (BW) was recorded 24 h prior to euthanasia, and the mice were housed individually in metabolic cages. Blood (~500 to 1,000 μL) was collected from each mouse by intracardiac puncture before euthanasia and centrifuged for serum. Urine (~100 to 400 μL/mouse)
was analyzed by albumin ELISA (Albuwell; Exocell, Philadelphia, PA) and a creatinine kit (Creatinine Companion; Exocell) (6–13,19).

After euthanization, the kidneys were removed, decapsulated, and weighed. Left kidneys were processed for histology and immunostaining, and right kidneys were harvested for isolation of RPTs by Percoll gradient (6–13,19). Aliquots of freshly isolated RPTs from individual mice were immediately processed for total RNA or protein analysis.

In separate experiments, adult male non-Akita WT mice (age 14 weeks) received an injection of either corn oil (5 mL/kg BW) or oltipraz (150 mg/kg/day i.p. in corn oil) with or without trigonelline (0.02 mg/kg/day i.p. in 0.9% NaCl) four times every other day (i.e., on days 1, 3, 5, and 7) according to published protocols (eight mice per group) (23, 24). Animals were killed 24 h after the last injection.

**Histology**

Tissue sections (four to five sections per kidney) were counterstained with periodic acid Schiff stain and analyzed by light microscopy by two investigators who were blinded to the treatments.

Immunostaining was performed with the standard avidin-biotin-peroxidase complex method on four to five sections per kidney and three mouse kidneys per group (ABC Staining; Santa Cruz Biotechnology, Santa Cruz, CA) (6–13,19).

Oxidative stress in RPTs was assessed by dihydroethidium (Sigma-Aldrich) staining in frozen kidney sections (13). The results were confirmed by standard assays of Cat activity (11), ROS generation (4,5,10–12), NADPH oxidase activity (10), and Nox4 mRNA expression (25).

**Real-Time Quantitative PCR**

Agt, Nrf2, Keap1, HO-1, and β-actin mRNA expression in RPTs was quantified by real-time quantitative PCR (RT-qPCR) with forward and reverse primers (Supplementary Table 1) (7–13,19).

**Western Blotting**

Western blotting (WB) was performed as described previously (6–13,19). The relative densities of Agt, Nrf2, Keap1, HO-1, and β-actin bands were quantified by densitometry using ImageQuant software (version 5.1; Molecular Dynamics, Sunnyvale, CA).

**Serum and Urinary Agt and Angiotensin II Measurement**

Serum and urinary Agt levels were quantified by ELISA (Immuno-Biological Laboratories, Minneapolis, MN) (8,10,13,19). To measure angiotensin II (Ang II) levels, serum and urine samples were extracted using a kit and were assayed by an ELISA specific for Ang II (Bachem, Torrance, CA) (8,10,13,19).

**Effect of HG, H₂O₂, and Oltipraz on Agt and Nrf2 Gene Expression in rRPTCs**

Immortalized rRPTCs (passages 12 through 18) (26) were studied. The plasmids pGL4.20-rAgt N-1495/+18 and pGL4.20-rNrf2 N-1980/+111 were stably transfected into rRPTCs (designated as stable transformants) (19).

To study the effects of HG, H₂O₂, and oltipraz, rRPTCs at 75–85% confluency or stable transformants were synchronized overnight in serum-free DMEM containing 5 mmol/L D-glucose, then incubated in medium containing 5 mmol/L D-glucose plus H₂O₂ (10⁻⁶ mol/L) or (5 mmol/L D-glucose plus 20 mmol/L D-mannitol) (normal glucose) in the absence or presence of oltipraz with or without trigonelline or HG (25 mmol/L D-glucose) DMEM containing 1% depleted FBS for 24 h in the presence of antioxidants, NF-κB inhibitors (PDTC or Bay 11-7082), the p38 MAPK inhibitor (SB203580), the p44/42 MAPK inhibitor (PD98059), or wortmannin (a phosphatidylinositol 3-kinase inhibitor) (2–5). Agt, HO-1, and Nrf2 mRNA levels were quantified by RT-qPCR, and corresponding Agt and Nrf2 gene promoter activity was measured by the luciferase activity assay (19). RPTCs stably transfected with the plasmid pGL4.20 served as controls.

In additional experiments, stable transformant RPTCs were transiently transfected with Nrf2 siRNA or scrambled siRNA, and the effects of HG on Nrf2 and Agt mRNA expression and their respective gene promoter activities were analyzed after 24 h of incubation.

**Statistical Analysis**

The data are expressed as mean ± SEM. Statistical comparisons were made by Student t test or one-way ANOVA and the Bonferroni test as appropriate. P values < 0.05 were considered to be statistically significant.

**RESULTS**

**Pathophysiological Measurements in Mice**

Supplementary Table 2 reports physiological measurements in non-Akita WT mice, Cat-Tg mice, Akita mice, and Akita Cat-Tg mice at week 16. Briefly, Cat overexpression had no effect on blood glucose levels, whereas it completely normalized SBP in Akita Cat-Tg compared with Akita mice. Cat overexpression markedly attenuated, but did not completely normalize, the GFR; urinary albumin/creatinine, kidney weight/tibia length, and heart weight/tibia length ratios; and urinary Agt and Ang II levels in Akita Cat-Tg compared with Akita mice. In contrast, Cat overexpression did not affect any of these parameters except for albumin/creatinine ratio and RPTC volume in Cat-Tg mice compared with WT controls.

**Histology**

Consistent with earlier observations (8,13,19), Akita mice developed renal structural damage (Supplementary Fig. 1A). The histologic changes include proximal tubule cell atrophy, tubular luminal dilatation with accumulation of cell debris, and increased extracellular matrix proteins in glomeruli and tubules. Cat overexpression markedly reversed but did not completely resolve these abnormalities in Akita Cat-Tg mice.

Cat immunostaining (Fig. 1A) and Cat activity (Fig. 1B) but not Cat mRNA expression (Fig. 1C) were significantly
lower in RPTs from Akita mice compared with non-Akita WT, Cat-Tg, Akita, and Akita Cat-Tg mouse kidneys with rabbit anti-bovine Cat polyclonal antibody. Magnification ×200. Cat activity (B), Cat mRNA (C), ROS generation (D), NADPH oxidase activity (E), and Nox4 mRNA (F) expression in RPTs of WT control, Cat-Tg, Akita, and Akita Cat-Tg mice. Values are expressed as mean ± SEM, n = 8 per group. *P < 0.05; **P < 0.01; ***P < 0.005. NS, not significant. RLU, relative luciferase unit.

**Figure 1**—Characterization of Akita Cat-Tg mice. A: Immunohistochemical staining for Cat in male non-Akita WT, Cat-Tg, Akita, and Akita Cat-Tg mouse kidneys with rabbit anti-bovine Cat polyclonal antibody. Magnification ×200. Cat activity (B), Cat mRNA (C), ROS generation (D), NADPH oxidase activity (E), and Nox4 mRNA (F) expression in RPTs of WT control, Cat-Tg, Akita, and Akita Cat-Tg mice. Values are expressed as mean ± SEM, n = 8 per group. *P < 0.05; **P < 0.01; ***P < 0.005. NS, not significant. RLU, relative luciferase unit.

**Effect of Cat Overexpression on Agt, Nrf2, HO-1, and Keap1 Expression in Akita Mice**

Immunostaining revealed significantly higher Agt (Fig. 2A), Nrf2 (Fig. 2B), and HO-1 expression (Fig. 2C) in RPTCs from Akita mice compared with non-Akita WT or Cat-Tg mice. Cat overexpression normalized Agt, Nrf2, and HO-1 expression in Akita Cat-Tg mice. In contrast, no significant differences in Keap1 expression were detected in RPTCs among the different groups (Fig. 2D). WB for Agt, Nrf2, Keap1, and HO-1 (Fig. 2Ea and b); and RT-qPCR for Agt, Nrf2, Keap1, and HO-1 mRNA expression (Fig. 2Fa-d, respectively) from isolated RPTs confirmed these findings. Furthermore, increases in nuclear Nrf2 levels but not cytoplasmic Nrf2 levels were observed in RPTs in Akita mice compared with WT and Cat-Tg mice. Cat overexpression attenuated the nuclear but not the cytoplasmic Nrf2 levels in Akita Cat-Tg mice (Fig. 2Ec).

**Effect of HG and H2O2 on Agt, Nrf2, and HO-1 Gene Expression in rRPTCs in Vitro**

Consistent with our previous observations on HG regulation of Agt gene expression (Supplementary Fig. 1B–E), HG stimulated Nrf2 mRNA in RPTCs in a concentration- and time-dependent manner (Fig. 3A and B, respectively). The HG stimulation of Nrf2 mRNA expression was inhibited by apocynin, diphenyleneiodonium chloride, rotenone, and Cat (Fig. 3C). Likewise, SB203580, but not PD98059 or wortmannin, prevented HG-stimulated increases in Nrf2 mRNA expression (Fig. 3D). Furthermore,
both PDTC and Bay 11-7082 inhibited HG-stimulated increases in Nrf2 and Agt at both the mRNA (Fig. 3E and F, respectively) and protein levels (Fig. 3G and H, respectively). Similarly, both Bay 11-7082 and PDTC inhibited HG stimulation of HO-1 expression at both the mRNA and protein levels (Fig. 3I and J, respectively).

We used H$_2$O$_2$ to determine whether ROS could directly stimulate Nrf2, Agt, and HO-1 gene expression in RPTCs. Indeed, H$_2$O$_2$ stimulated Nrf2, Agt, and HO-1 mRNA expression in RPTCs, and these effects were inhibited in the presence SB203580 and Bay 11-7082, but not wortmannin or PD98059 (Fig. 4A, B, and E, respectively). WB of HO-1 confirmed the HO-1 mRNA expression (Fig. 4F). Consistently, H$_2$O$_2$ stimulated Nrf2 and Agt gene promoter activity in RPTCs, and these were inhibited by SB203580 and Bay 11-7082, but not wortmannin and PD98059 (Fig. 4C and D, respectively). Most interestingly, transfection of pcDNA3.1 plasmid containing Flag-(Rel A) p65 cDNA stimulated Nrf2 and Agt gene promoter activity in a dose-dependent manner (Fig. 4G and H, respectively).
Figure 3 — Effect of HG on Nrf2, Agt, and HO-1 gene expression in rat RPTCs. Cells were incubated in various concentrations of D-glucose for 24 h (A) or for various time periods (B) in the presence of one of the following: antioxidants: apocynin (10⁻⁵ mol/L), diphenyleneiodonium chloride (DPI; 10⁻⁷ mol/L), Cat (300 units), or Rotenone (10⁻⁶ mol/L) (C); MAPK inhibitors: wortmannin (10⁻⁶ mol/L), PD98059 (10⁻⁵ mol/L), or SB203580 (10⁻⁶ mol/L) (D); or NF-κB inhibitors: Bay 11-7082 (10⁻⁵ mol/L) or PDTC (10⁻⁶ mol/L) (E and F). Cells were harvested and assayed for Nrf2 or Agt mRNA by RT-qPCR. WB of Nrf2 (G) and Agt (H) expression in RPTCs cultured in HG medium in the absence or presence of inhibitors Bay 11-7082 (10⁻⁵ mol/L) or PDTC (10⁻⁶ mol/L) (E and F). Cells were harvested and assayed for Nrf2 or Agt mRNA by RT-qPCR. WB of HO-1 protein (J) expression in RPTCs cultured in HG medium in the absence or presence of the NF-κB inhibitors Bay 11-7082 (10⁻⁵ mol/L) or PDTC (10⁻⁶ mol/L). Cells incubated in medium containing 5 mmol/L D-glucose plus 20 mmol/L D-mannitol were considered as controls (arbitrary unit 1). Results are reported as percentages of control values (mean ± SEM, n = 3). *P < 0.05; **P < 0.01; ***P < 0.005. NS, not significant. Similar results were obtained in two separate experiments.
Figure 4—Effect of H2O2 on Nrf2, Agt, and HO-1 mRNA expression in rat RPTCs. RPTCs or cells stably transfected with the pGL4.20 containing Nrf2 or Agt gene promoter were incubated with 10^{-6} mol/L H2O2 in 5 mmol/L D-glucose for 24 h in the absence or presence of wortmannin (10^{-6} mol/L), PD98059 (10^{-6} mol/L), SB203580 (10^{-6} mol/L), Bay 11-7082 (10^{-6} mol/L), or PDTC (10^{-6} mol/L). Cells were harvested and assayed for Nrf2 or Agt mRNA by RT-qPCR (A and B, respectively) or promoter activity of Nrf2 (C) and Agt (D) genes by luciferase activity assay or assayed for HO-1 mRNA (E) and HO-1 protein (F) by RT-qPCR and WB, respectively. Cells incubated in medium containing 5 mmol/L D-glucose were considered as controls (arbitrary unit 1). Results are expressed as relative values to control (mean ± SEM, n = 3). *P < 0.05; **P < 0.01; ***P < 0.005. NS, not significant. Similar results were obtained in two separate experiments. Effect of Flag-(Rel A)p65 cDNA transfection on Nrf (G) and Agt (H) gene promoter activity in RPTCs. Nrf2 and Agt gene promoter activity was quantified by luciferase activity assay. Luciferase activity in cells transfected with the plasmid pcDNA 3.1 was considered as a control (100%). Results are reported as percentages of control values (mean ± SEM, n = 3). *P < 0.05; **P < 0.01; ***P < 0.005. NS, not significant. RLU, relative luciferase unit.
Effect of Oltipraz and Trigonelline on Nrf2 and Agt Expression in Mice in Vivo

Immunostaining of RPTCs for Nrf2, Agt, and HO-1 revealed significantly higher expression levels in WT mice treated with oltipraz. Trigonelline coadministration reduced Nrf2, Agt, and HO-1 expression to levels similar to those of nontreated mice (Fig. 8A–C, respectively). Trigonelline alone had no detectable effects on the expression of either gene. WB for Nrf2, Agt, and HO-1 protein (Fig. 8D–F, respectively); and RT-qPCR of Nrf2, Agt, and HO-1 mRNA expression (Fig. 8G–I, respectively) confirmed these findings. Oltipraz also stimulated Nrf2 mRNA expression in the mouse liver, which was prevented by trigonelline (Supplementary Fig. 1G). Oltipraz, however, did not affect Agt mRNA expression in the liver (Supplementary Fig. 1H). These data demonstrate that Nrf2 activation differentially stimulates renal Agt gene expression and RAS activation in mice in vivo. Oltipraz enhanced ROS generation, and this was prevented by trigonelline coadministration (Supplementary Fig. 1I). However, no statistically significant differences in SBP were found between nontreated mice and mice treated with oltipraz with or without trigonelline (Supplementary Fig. 1J).

DISCUSSION

ROS generation, deficient antioxidant defenses, and dysregulation of RAS have long been implicated in the development of renal injury in diabetes. However, the underlying molecular mechanisms are far from being fully understood. Our present results document that selective Cat overexpression in RPTCs effectively suppresses Nrf2 stimulation of Agt gene expression, and attenuates systemic hypertension and kidney injury in Akita Cat-Tg mice. Nrf2 activation by oltipraz stimulates both Nrf2 and Agt gene expression in RPTCs, and the reversal of these actions by trigonelline or Nrf2 siRNA both in vitro and in vivo. These data indicate that Nrf2 activation by oxidative stress (secondary to hyperglycemia) stimulates intrarenal Agt gene expression and RAS activation, subsequently leading to hypertension and development of nephropathy, and identify a novel mechanism underlying the protective role of Cat.

In the Akita mouse, an autosomal-dominant model of spontaneous type 1 diabetes, the Ins2 gene, is mutated, closely resembling that in patients with type 1 diabetes (27,28). In the current study, we detected marked increases in ROS generation, NADPH oxidase activity, and Nox4 mRNA expression in RPTCs of Akita mice compared with non-Akita mice. These changes were normalized in Akita Cat-Tg mice, indicating that oxidative stress is a major component of renal injury in Akita mice.

Our data indicate that mitigating oxidative stress via kidney-specific Cat overexpression protects Akita mice against the development of hypertension. The mechanisms underlying elevated SBP in Akita mice are still largely unknown. Our present findings demonstrate significantly higher Agt expression in RPTCs, as well as higher urinary

Effect of Nrf2 Activation on Nrf2 and Agt Gene Expression in rRPTCs in Vitro

To study the impact of Nrf2 on Nrf2 and Agt gene expression in RPTCs, we used the Nrf2 activator oltipraz and the inhibitor alkaloid trigonelline, as well as Nrf2 cDNA transfection. Oltipraz stimulated Nrf2 and Agt mRNA expression (Fig. 5A and B, respectively) in a concentration-dependent manner. Trigonelline inhibited HG stimulation of Nrf2 and Agt mRNA expression (Fig. 5C and D, respectively) in a concentration-dependent manner. Transient transfection of Nrf2 cDNA stimulated both Nrf2 and Agt mRNA expression (Fig. 5E and F, respectively), as well as their respective gene promoters (Fig. 5G and H, respectively). Of note, Nrf2 mRNA levels in Fig. 5E were quantified with primers specific to the 3′-untranslated region of rat Nrf2 mRNA (Supplementary Table 1). These data demonstrated that Nrf2 activation stimulates both Nrf2 and Agt gene expression in RPTCs.

Next, we investigated the effects of Nrf2 knockdown on HG stimulation of Nrf2 and Agt gene expression in RPTCs. Transfection of RPTCs with Nrf2 siRNA reduced Nrf2 and Agt protein (Fig. 6A and B, respectively) and mRNA (Fig. 6C and D, respectively) expression in a concentration-dependent manner without affecting Keap1 mRNA expression (Supplementary Fig. 1P). Scrambled siRNA had no effect on Nrf2 and Agt expression. Nrf2 siRNA prevented HG stimulation of Nrf2 and Agt gene promoter activity (Fig. 6E and F, respectively) in a concentration-dependent manner.

Identification of Nrf2-REs in rAgt Gene Promoter

Figure 7A represents a schematic diagram of the rAgt gene promoter with putative Nrf2-REs. Two putative Nrf2-REs, agagccm and tgagccm, were localized in nucleotides N-964–N-959 and N-913–N-908 upstream of the transcription starting site of the rAgt gene promoter, and were designated as distal Nrf2-RE (dnRf2-RE) and proximal Nrf2-RE (pNrf2-RE), respectively. Transient transfection of plasmid containing nucleotides 1,495 and 1,031 upstream of the transcription starting sites pGL4.20 (Agt N-1495/+18) and pGL4.20 (Agt N-1031/+18) displayed 15-fold higher promoter activity than the promoterless plasmid pGL4.20 (Fig. 7B). Further deletion to N-442 of the rAgt gene promoter pGL4.20 (Agt N-442/+18) significantly reduced promoter activity compared with pGL4.20 (Agt N-1495/+18) and pGL4.20 (Agt N-1031/+18), indicating that enhancers might be localized within nucleotides N-1031 to N-443 of the rAgt gene promoter.

Oltipraz stimulated Agt gene promoter activity (pGL4.20 [Agt N-1495/+18]) (Fig. 7C). Trigonelline inhibited oltipraz stimulation of Agt gene promoter activity in a concentration-dependent fashion (Fig. 7D). Interestingly, the deletion of either dnRf2-RE or pNrf2-RE only partially attenuated the stimulatory effect of oltipraz, whereas the deletion of both Nrf2-REs completely abolished it (Fig. 7E). These data demonstrate that oltipraz induction of Agt gene transcription requires both Nrf2-REs in the rAgt gene promoter.

Identification of Nrf2-REs in rAgt Gene Promoter

Figure 7A represents a schematic diagram of the rAgt gene promoter with putative Nrf2-REs. Two putative Nrf2-REs, agagccm and tgagccm, were localized in nucleotides N-964–N-959 and N-913–N-908 upstream of the transcription starting site of the rAgt gene promoter, and were designated as distal Nrf2-RE (dnRf2-RE) and proximal Nrf2-RE (pNrf2-RE), respectively. Transient transfection of plasmid containing nucleotides 1,495 and 1,031 upstream of the transcription starting sites pGL4.20 (Agt N-1495/+18) and pGL4.20 (Agt N-1031/+18) displayed 15-fold higher promoter activity than the promoterless plasmid pGL4.20 (Fig. 7B). Further deletion to N-442 of the rAgt gene promoter pGL4.20 (Agt N-442/+18) significantly reduced promoter activity compared with pGL4.20 (Agt N-1495/+18) and pGL4.20 (Agt N-1031/+18), indicating that enhancers might be localized within nucleotides N-1031 to N-443 of the rAgt gene promoter.

Oltipraz stimulated Agt gene promoter activity (pGL4.20 [Agt N-1495/+18]) (Fig. 7C). Trigonelline inhibited oltipraz stimulation of Agt gene promoter activity in a concentration-dependent fashion (Fig. 7D). Interestingly, the deletion of either dnRf2-RE or pNrf2-RE only partially attenuated the stimulatory effect of oltipraz, whereas the deletion of both Nrf2-REs completely abolished it (Fig. 7E). These data demonstrate that oltipraz induction of Agt gene transcription requires both Nrf2-REs in the rAgt gene promoter.
Agt and Ang II levels in Akita mice than in non-Akita WT and Cat-Tg mice. Cat overexpression in RPTCs normalized these changes. These observations are consistent with the clinical findings of elevated intrarenal RAS gene expression in diabetic and hypertensive patients (29–33).

Consistent with reports that Cat activity and expression were downregulated in diabetic rats (34,35) and that hyperglycemia induced upregulation of Nrf2 expression in endothelial cells (36), we observed that Cat activity and expression were downregulated. While Akita mice
exhibited slightly decreased Cat mRNA expression, it did not differ significantly from that in WT mice. The reason for decreased Cat activity remains unclear. One possible explanation is that elevated ROS would result in decreased Cat activity without affecting the expression of Cat mRNA.

The precise mechanisms by which hyperglycemia leads to the upregulation of renal Nrf2 and Agt gene expression in diabetes remain unclear. One possibility is that ROS or hyperglycemia enhances Nrf2 activation via promoting its dissociation from Keap1 and translocation into the nucleus. Nrf2 would then bind to Nrf2-binding sites in the Agt gene promoter region and promote Agt gene expression. Indeed, our in vitro studies in rRPTCs confirmed that HG and oltipraz stimulate both Nrf2 and Agt gene expression; and that these can be reversed by trigonelline; Nrf2 siRNA; and pharmacological inhibitors of ROS, p38 MAPK, and NF-κB. Intriguingly, transient transfection of Nrf2 cDNA stimulated both Nrf2 and Agt mRNA and their respective gene promoter activities in RPTCs. This effect could be explained by the presence of Nrf2-REs in both Nrf2 (37) and Agt gene promoters (38). Furthermore, Nrf2 exerts positive auto-feedback on Nrf2 gene transcription (37). Consistently, the deletion of dNrf2-REs...
and pNrf2-REs completely abolished the stimulatory effect of oltipraz on Agt gene transcription, demonstrating that Nrf2 stimulation of Agt gene expression occurs at the transcriptional level.

In WT mice, the administration of oltipraz stimulated Nrf2, Agt, and HO-1 gene expression in RPTCs, and these actions were reversed by trigonelline coadministration. In contrast, oltipraz stimulated Nrf2 but not Agt gene expression in the liver. These findings highlight tissue-specific Nrf2 regulation of Agt gene expression.

The molecular mechanisms by which p38 MAPK and NF-κB signal Nrf2 and Agt gene expression are not fully understood. Further studies are needed to elucidate the precise signaling pathways involved.
understood. A likely mechanism is that HG activates p38 MAPK signaling via ROS generation, as we reported previously (3,4). Activated p38 MAPK would then phosphorylate Nrf2, resulting in dissociation from Keap1, as has been reported for various cell lines (39–41). Another possibility is that p38 MAPK activates NF-kB and increases the dissociation of NF-kB subunit p65 from subunit p50. The p65 subunit would then bind to Keap1 and release Nrf2, as reported in human embryonic kidney 293 cells (42). Alternatively, the activated p65 subunit likely translocates to the nucleus, binds to the NF-kB-REs in the Nrf2 and Agt gene promoter, and subsequently enhances Nrf2 and Agt gene transcription. Indeed, this possibility is supported by our observations that the transfection of the Rel A/p65 subunit stimulates gene promoter activity by both Nrf2 and Agt in RPTCs. Furthermore, consensus NF-kB-responsive DNA sequences 5’-GGG AAC TCC G-3’ and 5’-GGG ATT TCC C-3’ have been identified in the nucleotides N-371 to N-362 and N-578 to N-569 of the rat Nrf2 (37) and rat Agt gene promoter (38), respectively.

In contrast, Liu et al. (43) reported that NF-kB could directly repress Nrf2 signaling at the transcriptional level by competing with Nrf2 for transcription coactivator CREBP. We currently do not have an explanation for this discrepancy. Whether the Nrf2 released from the CREBP (caused by competitive binding of p65 on CREBP), as described by Liu et al. (43), could exert a “positive” feedback on Nrf2 gene transcription, as suggested by Kwak et al. (37), is unknown. Thus, further studies are needed to define the precise relationship of NF-kB signaling and Nrf2 gene transcription in RPTCs.

Studies in rodents with Nrf2 activators (bardoxolone methyl analogs RTA 405 and dh404) have yielded conflicting results. Bardoxolone methyl analogs were reported to have potent antidiabetes effects in diet-induced diabetic mice, and in rodent models of type 2 diabetes and obesity (44,45). In sharp contrast, recent studies (46) reported that bardoxolone methyl analogs increased albuminuria and blood pressure along with weight loss in Zucker diabetic fatty rats. In clinical trials,
phase 2 studies with bardoxolone methyl analogs reported reductions in serum creatinine levels or increases in the estimated GFR in human subjects with type 2 diabetes with stage 3b or 4 chronic kidney disease (47), suggesting a renoprotective action. However, phase 3 clinical trials involving patients with stage 4 advanced diabetic kidney disease were discontinued in 2012 after 9 months of follow-up because of increases in mortality rate and heart failure and the development of hypertension and albuminuria (48). Furthermore, the study that was discontinued did not show much slowing of the change in GFR (48). Our present data demonstrate that Nrf2 activation enhanced intrarenal Agt gene expression, suggesting that Nrf2 might exaggerate renal dysfunction via the activation of the intrarenal RAS.

In summary, our findings indicate ROS-evoked Nrf2-mediated Agt gene expression in diabetes models both in vivo and in vitro and document that these changes can be prevented by the selective overexpression of Cat in RPTCs. Our findings also imply an important role for oxidative stress–induced Nrf2 in the development of hypertension and renal injury in diabetes by altering the activation of the local intrarenal RAS.

**Funding.** This work was supported by grants from the Canadian Institutes of Health Research (MOP-97742 to J.G.F.; MOP-86450 to S.L.Z.; and MOP-84363, MOP 93650, and MOP 16088 to J.S.D.C.), the National Institutes of Health (HL-48455 to J.R.I.), and the Heart and Stroke Foundation of Canada (to J.S.D.C.). Editorial assistance was provided by the Centre Hospitalier de l’Université de Montréal Research Support Office and Ovid M. Da Silva.

**Duality of Interest.** No potential conflicts of interest relevant to this article were reported.

**Author Contributions.** S.A. and S.L.Z. researched the data and contributed to the discussion. Y.S., A.O., A.G., C.-S.L., and I.C. researched the data. J.G.F. and J.R.I. contributed to the discussion and reviewed and edited the manuscript. J.S.D.C. contributed to the discussion and wrote, reviewed, and edited the manuscript. J.S.D.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Prior Presentation.** Parts of this study were presented as a poster communication at Kidney Week 2013, the 46th Annual Meeting of the American Society of Nephrology, Atlanta, GA, 5–10 November 2013.

**References.**

1. Droz VJ, Ingelfinger JR. Molecular biology and pathophysiology of the intrarenal renin-angiotensin system. J Hypertens Suppl 1989;7:S3–S8
2. Zhang SL, Filep JG, Hohman TC, Tang SS, Ingelfinger JR, Chan JS. Molecular mechanisms of glucose action on angiotensinogen gene expression in rat proximal tubular cells. Kidney Int 1999;55:454–464
3. Zhang SL, Tang SS, Chen X, Filep JG, Ingelfinger JR, Chan JS. High levels of glucose stimulate angiotensinogen gene expression via the P38 mitogen-activated protein kinase pathway in rat kidney proximal tubular cells. Endocrinology 2000;141:4637–4646
4. Hsieh TJ, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, Chan JS. High glucose stimulates angiotensinogen gene expression via reactive oxygen species generation in rat kidney proximal tubular cells. Endocrinology 2002;143:2975–2985
5. Hsieh TJ, Fustier P, Zhang SL, et al. High glucose stimulates angiotensinogen gene expression and cell hypertrophy via activation of the hexosamine biosynthesis pathway in rat kidney proximal tubular cells. Endocrinology 2003;144:4338–4349
6. Sachettelli S, Liu Q, Zhang SL, et al. RAS blockade decreases blood pressure and proteinuria in transgenic mice overexpressing rat angiotensinogen gene in the kidney. Kidney Int 2006;69:1016–1023
7. Liu F, Brezniecanu ML, Wei CC, et al. Overexpression of angiotensinogen increases tubular apoptosis in diabetes. J Am Soc Nephrol 2008;19:269–280
8. Lo CS, Liu F, Shi Y, et al. Dual RAS blockade normalizes angiotensin-converting enzyme-2 expression and prevents hypertension and tubular apoptosis in Akta angiotensinogen-transgenic mice. Am J Physiol Renal Physiol 2012;302:F840–F852
9. Liu F, Wei CC, Wu SJ, et al. Apocynin attenuates tubular apoptosis and tubulointerstitial fibrosis in transgenic mice independent of hypertension. Kidney Int 2009;75:156–166
10. Godin N, Liu F, Lau GJ, et al. Catalase overexpression prevents hypertension and tubular apoptosis in angiotensinogen transgenic mice. Kidney Int 2010;77:1086–1097
11. Brezniecanu ML, Liu F, Wei CC, et al. Catalase overexpression attenuates angiotensinogen expression and apoptosis in diabetic mice. Kidney Int 2007;71:912–923
12. Brezniecanu ML, Liu F, Wei CC, et al. Attenuation of interstitial fibrosis and tubular apoptosis in db/db transgenic mice overexpressing catalase in renal proximal tubular cells. Diabetes 2008;57:451–459
13. Shi Y, Lo C-S, Chenier I, et al. Overexpression of catalase prevents hypertension and tubulointerstitial fibrosis and normalization of renal angiotensin-converting enzyme-2 expression in Akta mice. Am J Physiol Renal Physiol 2013;304:F1335–F1346
14. Sury TJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. Planta Med 2008;74:1526–1539
15. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol Med 2004;10:549–557
16. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A 1996;93:14960–14965
17. Wang L, Lei C, Zhang SL, et al. Synergistic effect of dexamethasone and isoproterenol on the expression of angiotensinogen in immortalized rat proximal tubular cells. Kidney Int 1996;53:287–295
18. Ding Y, Sigmund CD. Androgen-dependent regulation of human angiotensinogen expression in KAP-hAGT transgenic mice. Am J Physiol Renal Physiol 2001;280:F54–F60
19. Abdo S, Lo CS, Chenier I, et al. Heterogeneous nuclear ribonucleoproteins F and K mediate insulin inhibition of renal angiotensinogen gene expression and prevention of hypertension and kidney injury in diabetic mice. Diabetologia 2013;56:1649–1660
20. Chan K, Lu R, Chang JC, Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci U S A 1996:93:13943–13948
21. Chen X, Zhang SL, Pang L, et al. Characterization of a putative insulin-responsive element and its binding protein(s) in rat angiotensinogen gene promoter: regulation by glucose and insulin. Endocrinology 2001;142:2577–2585
22. Qi Z, Fujita H, Jin J, et al. Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. Diabetes 2005;54:2628–2637
23. Aleksunes LM, Reisman SA, Yeager RL, Goedken MJ, Klaassen CD. Nuclear factor erythroid 2-related factor 2 deletion impairs glucose tolerance and exacerbates hyperglycemia in type 1 diabetic mice. J Pharmacol Exp Ther 2010;333:140–151
24. Artt A, Sebens S, Krebs S, et al. Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity. Oncogene 2013;32:4825–4835
25. Diebold I, Petry A, Hess J, Gürtach A. The NADPH oxidase subunit NOX4 is a new target gene of the hypoxia-inducible factor-1. Mol Biol Cell 2010;21:2087–2096

26. Ingelfinger JR, Jung F, Diamant D, et al. Rat proximal tubule cell line transformed with origin-defective SV40 DNA: autocrine ANG II feedback. Am J Physiol 1999;276:F218–F227

27. Yoshioka M, Kayo T, Ikeda T, Kuizumi A. A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. Diabetes 1997;46:887–894

28. Haseyama T, Fujita T, Hirasawa F, et al. Complications of IgA nephropathy in a non-insulin-dependent diabetes model, the Akita mouse. Tohoku J Exp Med 2002;198:233–244

29. Lai KN, Leung JCK, Lai KB, To WY, Yeung VTF, Lai FMM. Gene expression of the renin-angiotensin system in human kidney. J Hypertens 1998;16:91–102

30. Navar LG, Harrison-Bernard LM, Nishiyama A, Kobori H. Regulation of intrarenal angiotensin II in hypertension. Hypertension 2002;39:316–322

31. Saito T, Urushihara M, Kotani Y, Kagami S, Kobori H. Increased urinary angiotensinogen is precedent to increased urinary albumin in patients with type 1 diabetes. Am J Med Sci 2009;338:478–480

32. Kobori H, Urushihara M, Xu JH, Berenson GS, Navar LG. Urinary angiotensinogen is correlated with blood pressure in men (Bogalusa Heart Study). J Hypertens 2010;28:1422–1428

33. Navar LG, Prieto MC, Satou R, Kobori H. Intrarenal angiotensin II and its contribution to the genesis of chronic hypertension. Curr Opin Pharmacol 2011;11:180–186

34. Celik S, Akkaya H. Total antioxidant capacity, catalase and superoxide dismutase on rats before and after diabetes. J Anim Vet Adv 2009;8:1503–1508

35. Takemoto K, Tanaka M, Iwata H, et al. Low catalase activity in blood is associated with the diabetes caused by alloxan. Clin Chim Acta 2009;407:43–46

36. Ungvari Z, Bailey-Downs L, Gautam D, et al. Adaptive induction of NF-E2-related factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia. Am J Physiol Heart Circ Physiol 2011;300:H1133–H1140

37. Kwak MK, Itoh K, Yamamoto M, Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. Mol Cell Biol 2002;22:2883–2892

38. Chan JS, Chan AHH, Jiyan G, Nie ZR, LaChance S, Carrière S. Molecular cloning and expression of the rat angiotensinogen gene. Pediatr Nephrol 1990;4:429–435

39. Gong P, Hu B, Cederbaum AI. Diallyl sulfide induces heme oxygenase-1 through MAPK pathway. Arch Biochem Biophys 2004;432:252–260

40. Shan Y, Wang X, Wang W, He C, Bao Y. p38 MAPK plays a distinct role in sulfuraphane-induced up-regulation of ARE-dependent enzymes and down-regulation of COX-2 in human bladder cancer cells. Oncol Rep 2010;23:1133–1138

41. Chen XQ, Wu SH, Zhou Y, Tang YR. Lipoxin A4-induced heme oxygenase-1 protects cardiomyocytes against hypoxia/reoxygenation injury via p38 MAPK activation and Nrf2/ARE complex. PLoS One 2013;8:e67120

42. Yu M, Li H, Liu Q, et al. Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. Cell Signal 2011;23:883–892

43. Liu GH, Qu J, Shen X. NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to Nrf2. Biochim Biophys Acta 2008;1783:713–727

44. Saha PK, Reddy VT, Konopleva M, Andreff M, Chan L. The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic-acid methyl ester has potent anti-diabetic effects in diet-induced diabetic mice and Lepr(db/db) mice. J Biol Chem 2010;285:40581–40592

45. Chin M, Lee CY, Chung JC, et al. Bardoxolone methyl analogs RTA 405 and db404 are well tolerated and exhibit efficacy in rodent models of type 2 diabetes and obesity. Am J Physiol Renal Physiol 2013;304:F1438–F1446

46. Zoja C, Corna D, Nava V, et al. Analogs of bardoxolone methyl worsen diabetic nephropathy in rats with additional adverse effects. Am J Physiol Renal Physiol 2013;304:F808–F819

47. Pergola PE, Raskin P, Toto RD, et al.; BEAM Study Investigators. Bardoxolone methyl and kidney function in CKD with type 2 diabetes. N Engl J Med 2013;365:327–336

48. de Zeeuw D, Akizawa T, Audhya P, et al.; BEACON Trial Investigators. Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. N Engl J Med 2013;369:2492–2503