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

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COMPLICATIONS

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 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

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hypertension and RPTC apoptosis in nondiabetic 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 relation 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 (H_2O_2), 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 trigonelline coadministration.

RESEARCH DESIGN AND METHODS

Chemicals and Constructs

D-Glucose, D-mannitol, H_2O_2, 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 D-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 r*Agt* (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 r*Cat* cDNA fused with HA-tag (which encodes amino acid residues 98–106 of human influenza virus hemagglutinin at the carboxyl terminus with the *Not*I site at both the 5′ and 3′ termini) was inserted into plasmid pKAP2 at the *Not*I site (11). The r*Agt* 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 r*Agt* gene promoter (21). Supplementary Table 1 details the oligo primers for cloning of the r*Agt* and r*Nrf2* 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-*Ins2*^{Akita}/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, H_2O_2, and Oltipraz on Agt and Nrf2 Gene Expression in rRPTCs

Immortalized rRPTCs (passages 12 through 18) (26) were studied. The plasmids pGL4.20-r*Agt N-1495/+18* and pGL4.20-r*Nrf2 N-1980/+111* were stably transfected into rRPTCs (designated as stable transformants) (19).

To study the effects of HG, H_2O_2, 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 H_2O_2 (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. 1*A*). 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. 1*A*) and Cat activity (Fig. 1*B*) but not *Cat* mRNA expression (Fig. 1*C*) were significantly

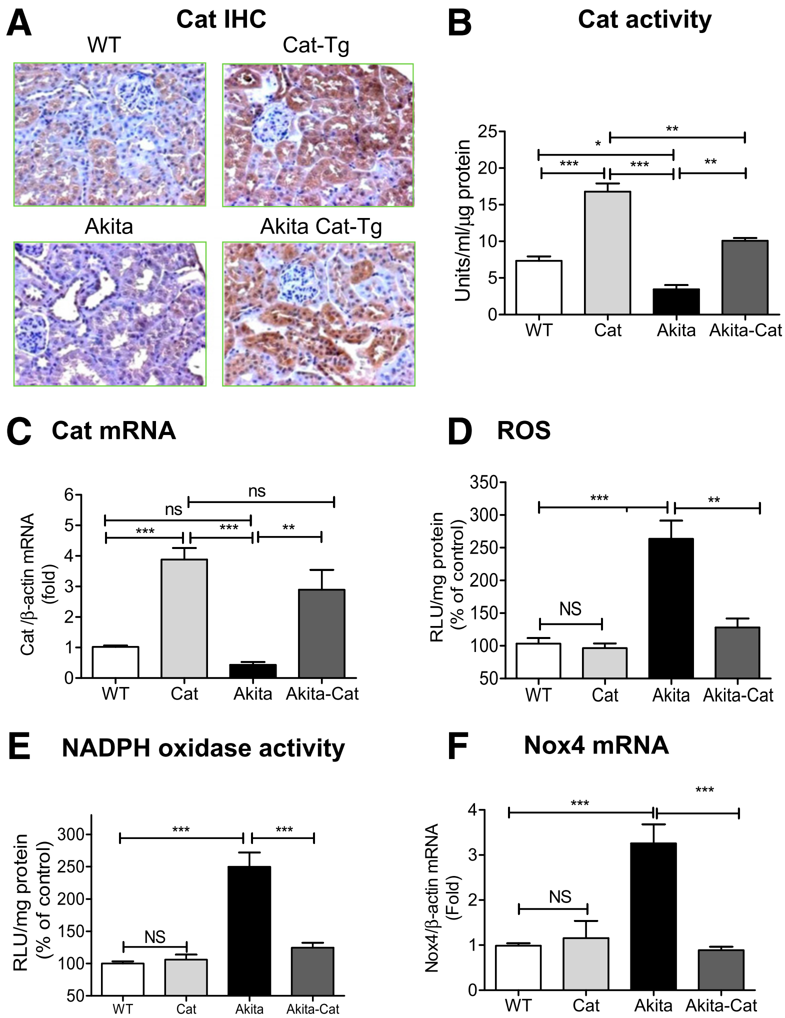

Figure 1—Characterization of Akita Cat-Tg mice. *A*: Immunohistochemical staining for Cat in male non-Akita WT, Cat-Tg, Akita, and Akita Cat-Tg mouse kidneys with rabbit anti-bovine Cat polyclonal antibody. Magnification ×200. Cat activity (*B*), Cat mRNA (*C*), ROS generation (*D*), NADPH oxidase activity (*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.

lower in RPTs from Akita mice compared with non-Akita WT, Cat-Tg, or Akita Cat-Tg mice. In contrast, Akita mice exhibited significantly higher ROS levels, quantified by lucigenin assay (Fig. 1*D*), NADPH oxidase activity (Fig. 1*E*), and *Nox4* mRNA, quantified by RT-qPCR (Fig. 1*F*), than non-Akita WT and Cat-Tg mice, indicating the presence of markedly higher levels of oxidative stress. These changes were normalized in Akita Cat-Tg mice.

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

Immunostaining revealed significantly higher Agt (Fig. 2*A*), Nrf2 (Fig. 2*B*), and HO-1 expression (Fig. 2*C*) 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. 2*D*). WB for Agt, Nrf2, Keap1, and HO-1 (Fig. 2*Ea* and *b*); and RT-qPCR for *Agt*, *Nrf2*, *Keap1*, and *HO-1* mRNA expression (Fig. 2*Fa–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. 2*Ec*).

Effect of HG and H_2O_2 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. 1*B–E*), HG stimulated *Nrf2* mRNA in RPTCs in a concentration- and time-dependent manner (Fig. 3*A* and *B*, respectively). The HG stimulation of *Nrf2* mRNA expression was inhibited by apocynin, diphenyleneiodonium chloride, rotenone, and Cat (Fig. 3*C*). Likewise, SB203580, but not PD98059 or wortmannin, prevented HG-stimulated increases in *Nrf2* mRNA expression (Fig. 3*D*). Furthermore,

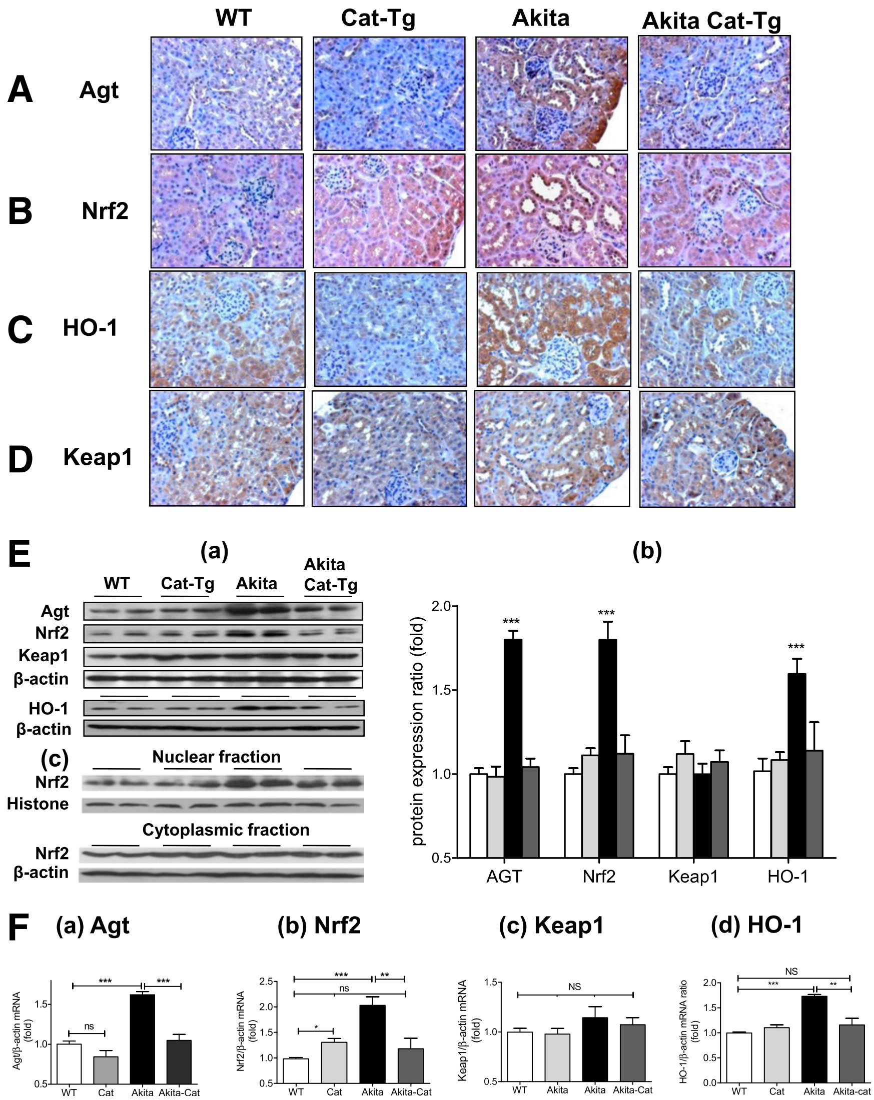

Figure 2—Agt, Nrf2, HO-1, and Keap1 expression in Tg mouse kidneys at week 16. Immunohistochemical staining for Agt (*A*), Nrf2 (*B*), HO-1 (*C*), and Keap1 (*D*) in mouse kidneys. Magnification ×200. *E*: WB of Agt, Nrf2, Keap1, and HO-1 expression (*a*) and quantification of their expression (*b*) in RPTs from kidneys of WT control, Cat-Tg, Akita, and Akita Cat-Tg mice. The membranes were reblotted for β-actin. Agt, Nrf2, Keap1, and HO-1 levels were normalized by corresponding β-actin levels. Values are expressed as mean ± SEM (n = 8). ***P < 0.005. *Ec*: WB of Nrf2 in nuclear and cytoplasmic fraction of RPTs from kidneys of WT control, Cat-Tg, Akita, and Akita Cat-Tg mice. NE-PER Nuclear and Cytoplasmic Extraction Reagents were used for the isolation of cytoplasmic and nuclear fractions from freshly isolated mouse RPTs according to the manufacturer's protocol (catalog no. 78833 [Pierce Protein Biology Products]; Thermo Scientific, Rockford, IL). *F*: RT-qPCR of *Agt* (*a*), *Nrf2* (*b*), *Keap1* (*c*), and *HO-1* (*d*) mRNA expression in RPTs of WT control, Cat-Tg, Akita, and Akita Cat-Tg mice. *Agt*, *Nrf2*, *Keap1*, and *HO-1* mRNA levels were normalized by corresponding β-actin mRNA levels. mRNA levels in non-Akita control littermates were considered as arbitrary unit 1. Values are reported as mean ± SEM, n = 8. **P < 0.01; ***P < 0.005. NS, not significant. WT (empty bars), Cat-Tg mice (light gray bars), Akita (solid black bars), and Akita Cat-Tg mice (dark gray bars).

both PDTC and Bay 11-7082 inhibited HG-stimulated increases in *Nrf2* and *Agt* at both the mRNA (Fig. 3*E* and *F*, respectively) and protein levels (Fig. 3*G* 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. 3*I* and *J*, respectively).

We used H_2O_2 to determine whether ROS could directly stimulate *Nrf2*, *Agt*, and *HO-1* gene expression in RPTCs. Indeed, H_2O_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. 4*A*, *B*, and *E*, respectively). WB of HO-1 confirmed the HO-1 mRNA expression (Fig. 4*F*). Consistently, H_2O_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. 4*C* 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. 4*G* and *H*, respectively).

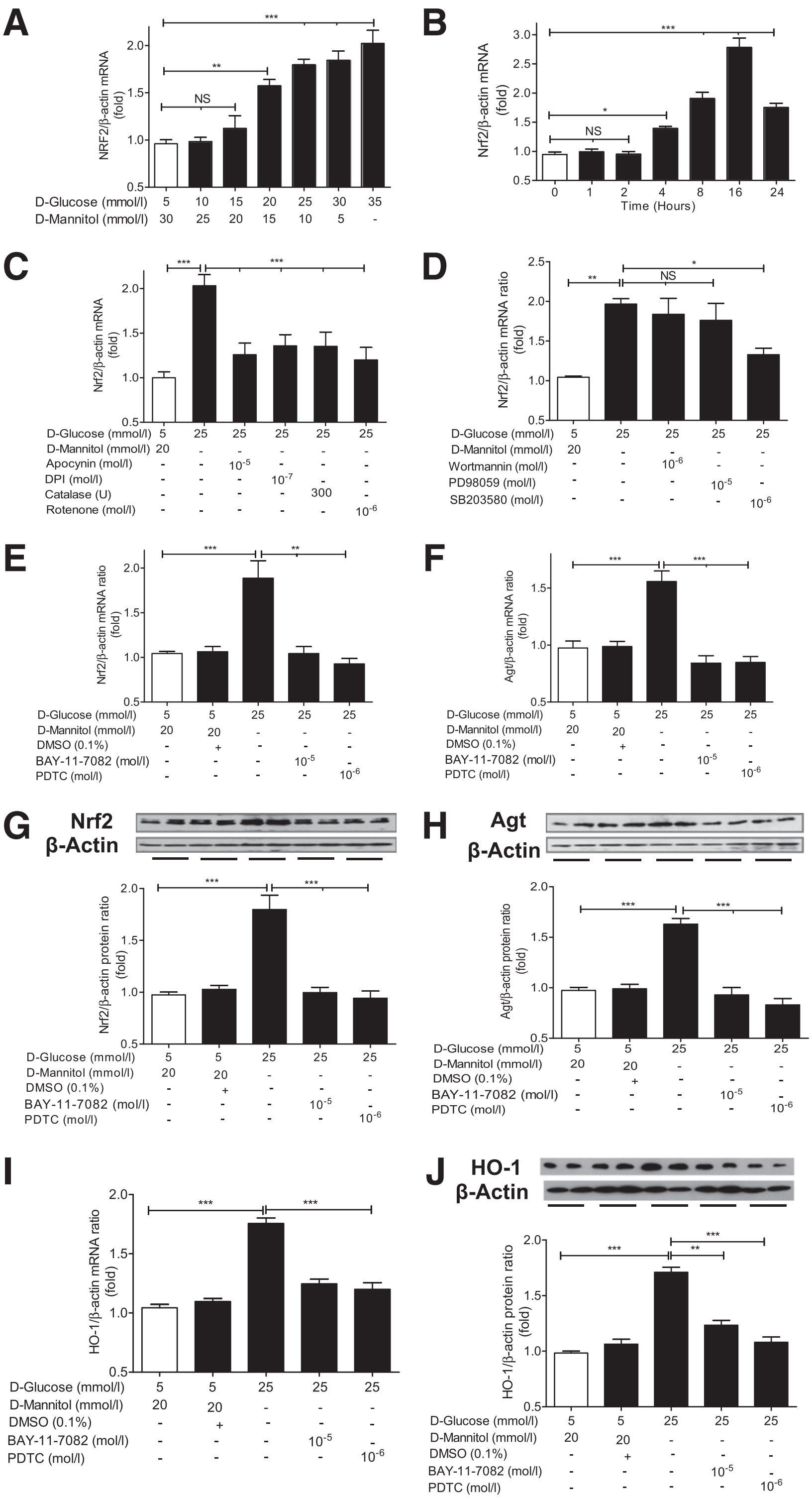

Figure 3—Effect of HG on *Nrf2*, *Agt*, and *HO-1* gene expression in rat RPTCs. Cells were incubated in various concentration of D-glucose for 24 h (*A*) or for various time periods (*B*) in the presence of one of the following: antioxidants: apocynin (10^{-5} mol/L), diphenyleneiodonium chloride (DPI; 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). RT-qPCR of *HO-1* mRNA (*I*) and 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.

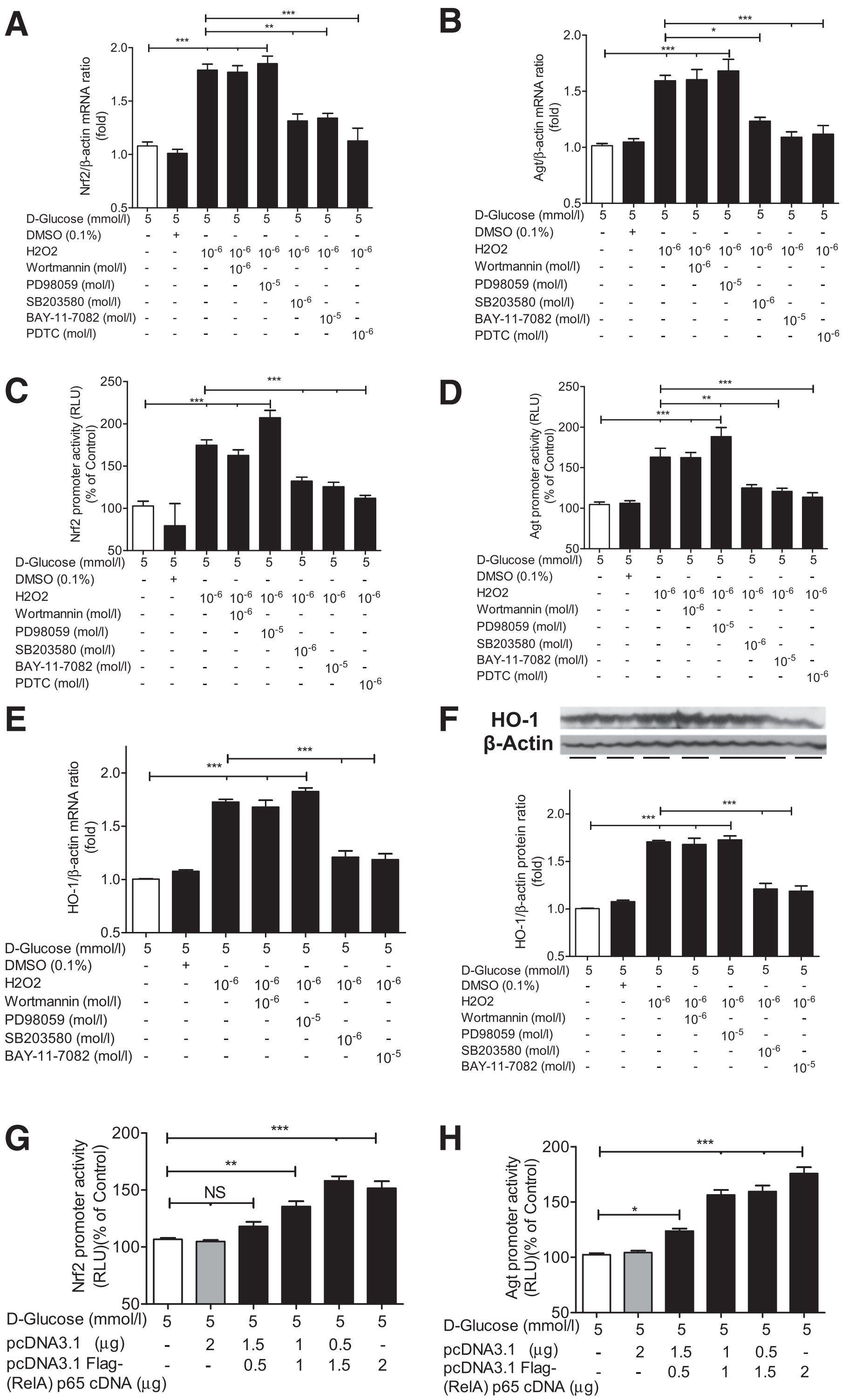

Figure 4—Effect of H_2O_2 on *Nrf2*, *Agt*, and *HO-1* mRNA expression in rat RPTCs. RPTCs or cells stably transfected with the pGL4.20 containing *Nrf2* or *Agt* gene promoter were incubated with 10^{-6} mol/L H_2O_2 in 5 mmol/L D-glucose for 24 h in the absence or presence of wortmannin (10^{-6} mol/L), PD98059 (10^{-5} mol/L), SB203580 (10^{-6} mol/L), Bay 11-7082 (10^{-5} 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 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. 5*A* and *B*, respectively) in a concentration-dependent manner. Trigonelline inhibited HG stimulation of *Nrf2* and *Agt* mRNA expression (Fig. 5*C* and *D*, respectively) in a concentration-dependent manner. Transient transfection of *Nrf2* cDNA stimulated both *Nrf2* and *Agt* mRNA expression (Fig. 5*E* and *F*, respectively), as well as their respective gene promoters (Fig. 5*G* and *H*, respectively). Of note, *Nrf2* mRNA levels in Fig. 5*E* 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. 6*A* and *B*, respectively) and mRNA (Fig. 6*C* and *D*, respectively) expression in a concentration-dependent manner without affecting Keap1 mRNA expression (Supplementary Fig. 1*F*). Scrambled siRNA had no effect on Nrf2 and Agt expression. *Nrf2* siRNA prevented HG stimulation of *Nrf2 and Agt* gene promoter activity (Fig. 6*E* and *F*, respectively) in a concentration-dependent manner.

Identification of Nrf2-REs in r*Agt* Gene Promoter

Figure 7*A* represents a schematic diagram of the *rAgt* gene promoter with putative *Nrf2*-REs. Two putative *Nrf2*-REs, *agagccnn* and *tgagccnn*, 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 (d*Nrf2*-RE) and proximal *Nrf2*-RE (p*Nrf2*-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. 7*B*). Further deletion to *N*-442 of the r*Agt* 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 r*Agt* gene promoter.

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

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 those of nontreated mice (Fig. 8*A*–*C*, respectively). Trigonelline alone had no detectable effects on the expression of either gene. WB for Nrf2, Agt, and HO-1 protein (Fig. 8*D*–*F*, respectively); and RT-qPCR of *Nrf2*, *Agt*, and *HO-1* mRNA expression (Fig. 8*G*–*I*, respectively) confirmed these findings. Oltipraz also stimulated *Nrf2* mRNA expression in the mouse liver, which was prevented by trigonelline (Supplementary Fig. 1*G*). Oltipraz, however, did not affect *Agt* mRNA expression in the liver (Supplementary Fig. 1*H*). 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. 1*I*). However, no statistically significant differences in SBP were found between nontreated mice and mice treated with oltipraz with or without trigonelline (Supplementary Fig. 1*J*).

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

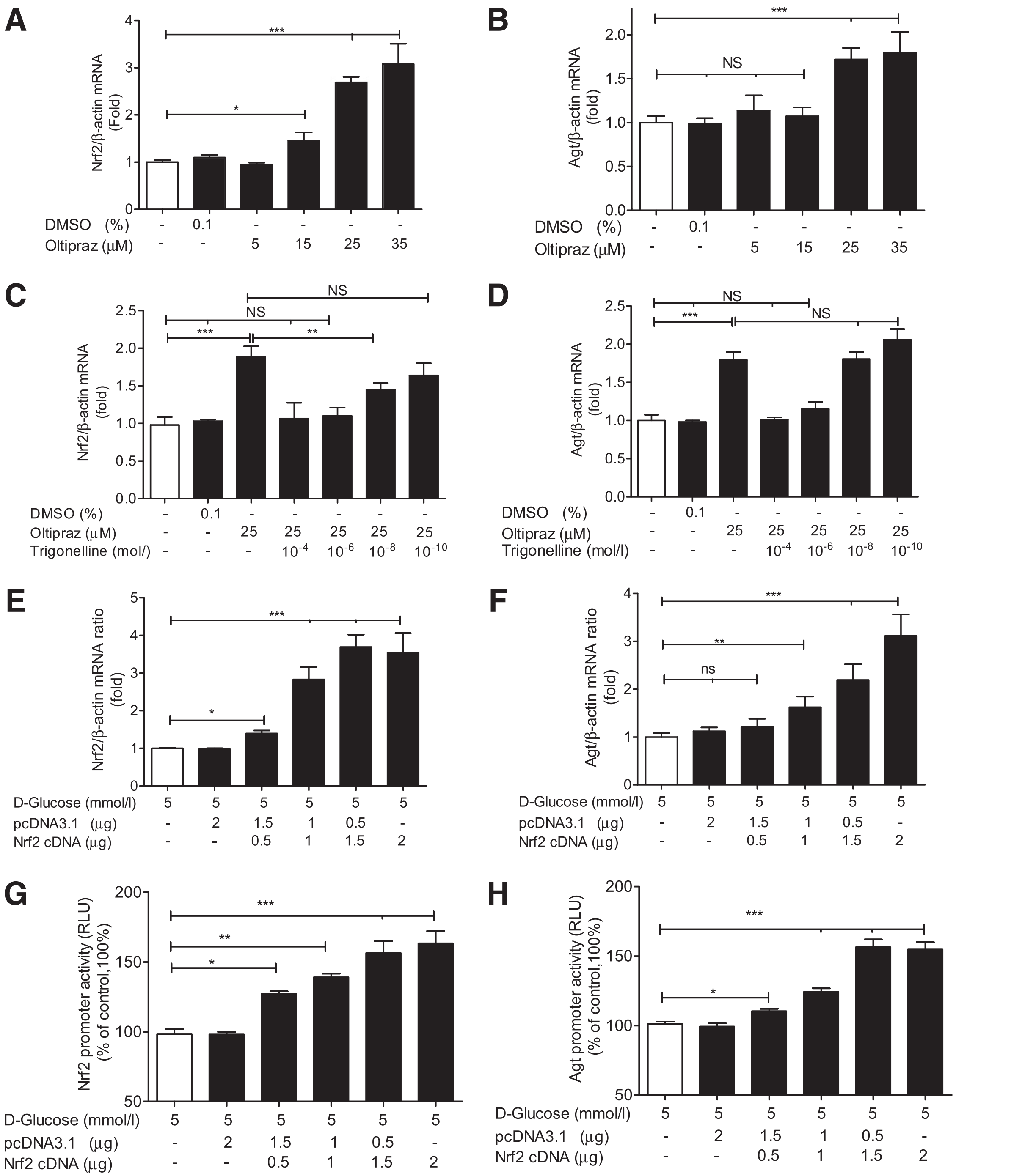

Figure 5—Effect of Nrf2 activator on *Nrf2* and *Agt* gene expression in rat RPTCs. Cells were incubated in various concentrations of the Nrf2 activator oltipraz for 24 h (*A* and *B*) in the absence or presence of various concentrations of the Nrf2 inhibitor alkaloid trigonelline (*C* and *D*). Effect of *Nrf2* cDNA transfection on *Nrf* (*E*) and *Agt* mRNA (*F*) expression in RPTCs. Cells were harvested and assayed for *Nrf2* or *Agt* mRNA by RT-qPCR. Effect of *Nrf2* 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. Cells incubated in a medium containing 5 mmol/L D-glucose were considered as controls (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. Similar results were obtained in two separate experiments.

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

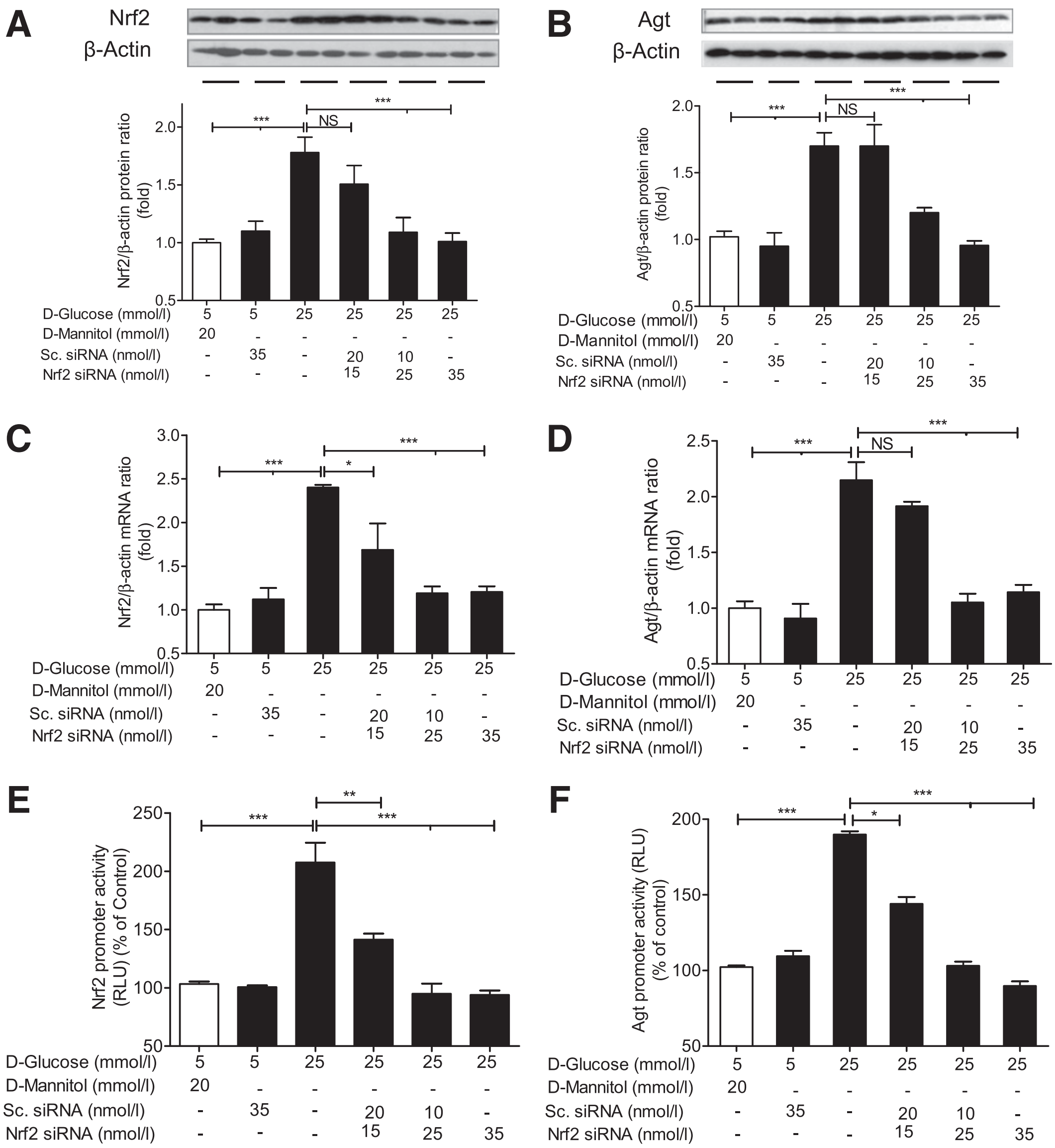

Figure 6—Effect of *Nrf2* siRNA on *Nrf2* and *Agt* gene expression in RPTCs in HG medium. Dose-dependent effect of *Nrf2* siRNA or scrambled (Sc.) siRNA on Nrf2 (*A*) and Agt (*B*) protein expression in RPTCs incubated in HG medium and quantified by WB. Dose-dependent effect of *Nrf2* siRNA or scrambled siRNA on *Nrf2* (*C*) and *Agt* (*D*) mRNA expression in RPTCs incubated in HG medium and quantified by RT-qPCR. Dose-dependent effect of *Nrf2* siRNA or scrambled siRNA on *Nrf2* (*E*) and *Agt* (*F*) gene promoter activity in RPTCs incubated in HG medium was quantified by luciferase activity assay. Cells were harvested after 24 h of incubation. *Agt* mRNA levels in cells incubated in normal glucose medium are expressed as arbitrary unit 1. The 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.

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 d*Nrf2*-REs

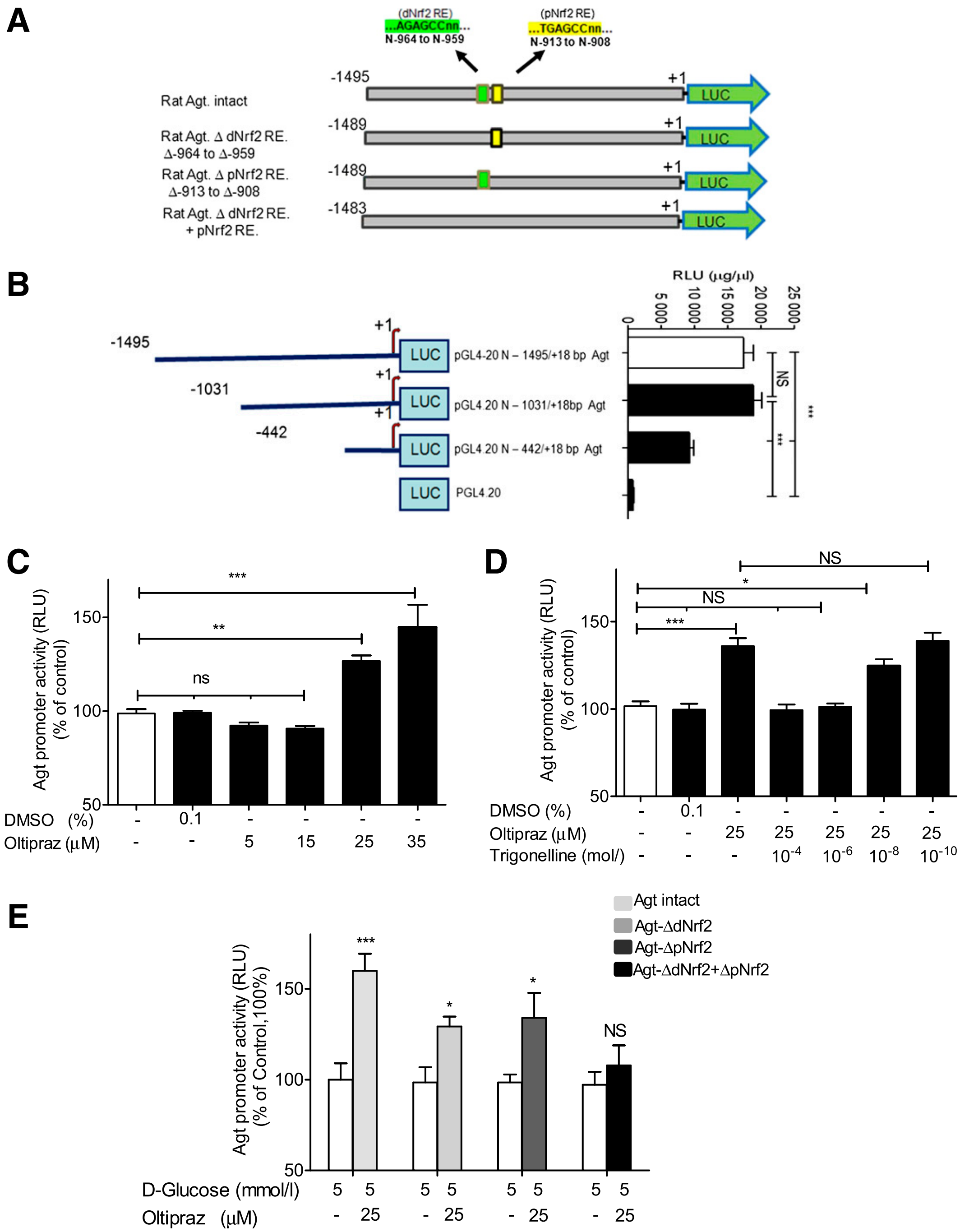

Figure 7—Idenfication of *Nrf2*-REs in the Agt gene promoter. *A*: Schematic diagram of *rAgt* gene promoter with putative *Nrf2*-REs. *B*: Activity of plasmid containing various lengths of *Agt* gene promoter assayed by luciferase (LUC) assay. Concentration-dependent effects of the Nrf2 activator oltipraz on *Agt* gene promoter activity in RPTCs in 5 mmol/L D-glucose medium in the absence (*C*) or presence (*D*) of the Nrf2 inhibitor trigonelline. *E*: Effect of oltipraz on full-length *Agt* gene promoter activity with or without *Nrf2*-RE (d*Nrf2*-RE, p*Nrf2*-RE, or both). The cells were incubated in medium containing 5 mmol/L D-glucose for 24 h and then harvested. The results are reported as percentages of control values (mean ± SEM), $n = 3$ independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. NS, not significant; RLU, relative luciferse unit.

and p*Nrf2*-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

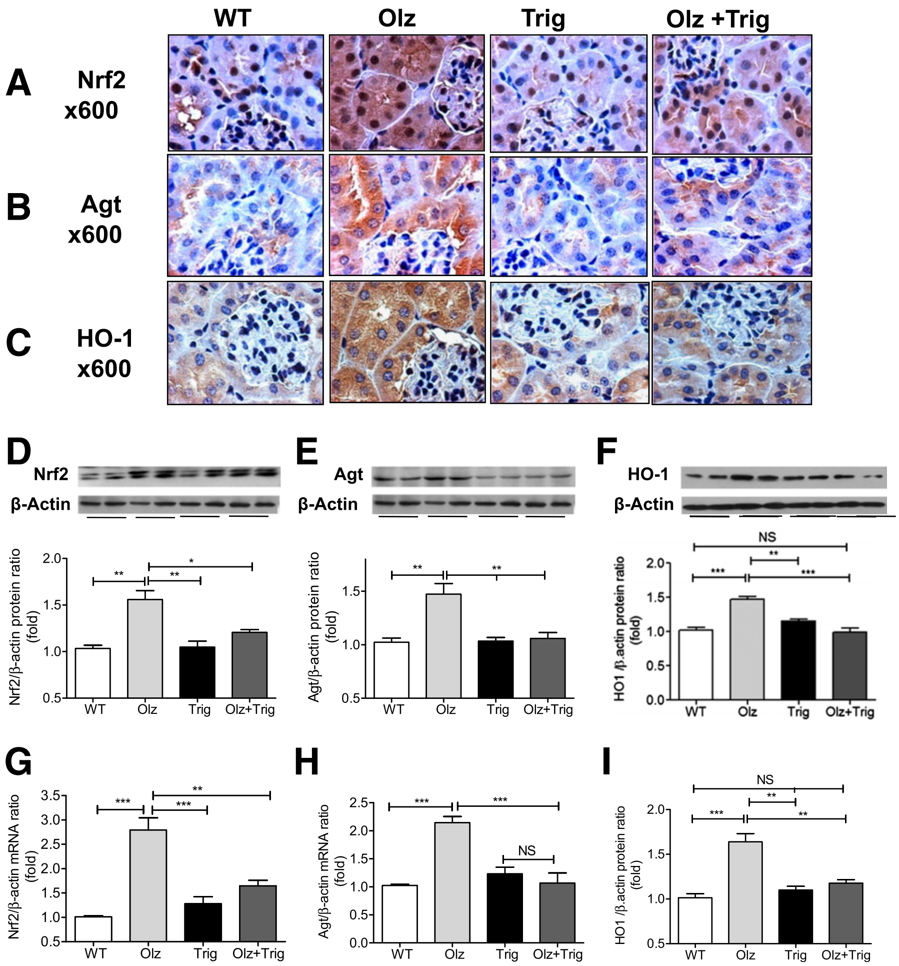

Figure 8—Effect of oltipraz on *Nrf2*, *Agt*, and *HO-1* mRNA expression in mice in vivo. Immunohistochemical staining for Nrf2 (*A*), Agt (*B*), and HO-1 (*C*) in the kidneys of WT mice with or without oltipraz (Olz) and trigonelline (Trig). Magnification ×200. WB of Nrf2 (*D*), Agt (*E*), and HO-1 (*F*) expression and RT-qPCR of *Nrf2* (*G*), *Agt* (*H*), and *HO-1* (*I*) mRNA expression in RPTs of WT mice with or without oltipraz and trigonelline. Values are expressed as mean ± SEM (n = 6 per group). *P < 0.05; **P < 0.01; ***P < 0.005. NS, not significant.

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-κB and increases the dissociation of NF-κB 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-κB-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-κB–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-κB 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-κB 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.

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