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Catalase overexpression attenuates angiotensinogen expression and apoptosis in diabetic mice

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Increased generation of reactive oxygen species (ROS) leads to oxidative stress in diabetes. Catalase is a highly conserved heme-containing protein that reduces hydrogen peroxide to water and oxygen and is an important factor decreasing cellular injury owing to oxidative stress. Hyperglycemic conditions increase oxidative stress and angiotensinogen gene expression. Angiotensinogen conversion to angiotensin II leads to a furtherance in oxidative stress through increased generation of reactive oxygen species. In this study, we utilized mice transgenically overexpressing rat catalase in a kidney-specific manner to determine the impact on ROS, angiotensinogen and apoptotic gene expression in proximal tubule cells of diabetic animals. Proximal tubules isolated from wild-type and transgenic animals without or with streptozotocin-induced diabetes were incubated in low glucose media in the absence or presence of angiotensin II or in a high-glucose media. Our results show that the overexpression of catalase prevents the stimulation of ROS and angiotensinogen mRNA in tubules owing to elevated glucose or angiotensin II *in vitro*. Additionally, overexpression of catalase attenuated ROS generation, angiotensinogen and proapoptotic gene expression and apoptosis in the kidneys of diabetic mice *in vivo*. Our studies point to an important role of ROS in the pathophysiology of diabetic nephropathy.

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Oxidative stress is defined as tissue injury induced by increased reactive oxygen species (ROS) generation. The key initial step in ROS formation is the conversion of molecular oxygen (O_2) to superoxide ($O_2^{\bullet -}$). Several enzymatic pathways can generate $O_2^{\bullet -}$, but in quantitative terms, the electron transport chain in mitochondria and nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase in membrane are the most important sources in both physiological and pathological conditions (reviewed in references[1–5]). Superoxide dismutases are key enzymes that neutralize $O_2^{\bullet -}$ into less reactive hydrogen peroxide (H_2O_2) which is then reduced to H_2O by catalase (CAT) or glutathione peroxidase.

Mammalian CAT is a tetrameric 240-kDa heme-containing protein that is highly conserved in humans, rats, and mice.[6–8] CAT expression is highly tissue-specific with the highest levels being found in the liver, kidney, and blood.[9–11] In the kidney, CAT is localized predominantly in the cytoplasm of proximal tubules of the juxtamedullary cortex, with a lesser amount in proximal tubules of the superficial cortex. It is not detected in the glomeruli, distal tubules, loop of Henle, and collecting ducts.[12]

The role of CAT in defending against cell and tissue injury by oxidative stress has been studied extensively. CAT overexpression renders cells more resistant to H_2O_2 toxicity and oxidant-mediated injury.[13,14] Transgenic (Tg) mice overexpressing CAT are protected against myocardial injury in hypertension.[15,16] In contrast, CAT knockout or null mice are phenotypically normal,[17] without those clinical manifestations of acatalasemia as seen in humans including oral ulcers and periodic hemolytic episodes.[18] However, CAT-null mice show differential sensitivity to oxidant-mediated tissue injury.[17] H_2O_2 is also known to control cell proliferation via modulation of cell signalling.[19] It is postulated that CAT overexpression might modulate H_2O_2 levels and thereby alter specific gene expression and cellular function in a tissue-specific manner.

Glomerular damage is a hallmark of renal injury in diabetes.[20] However, a number of studies reported that tubular atrophy might be a better predictor of renal disease

progression than glomerular pathology.[21–23] Both tubular atrophy and interstitial fibrosis are closely associated with loss of renal function.[24,25] Furthermore, 71% of glomeruli from proteinuric type I diabetic patients have glomerulotubular junction abnormalities, including 8–17% atubular glomeruli,[26,27] indicating that proximal tubular atrophy contributes significantly to renal failure in diabetes.

The mechanisms underlying tubular atrophy are not well understood. One attractive mechanism is apoptosis, which has been demonstrated to mediate cell death in a variety of renal diseases including diabetes.[28–33] We reported previously that high glucose evokes ROS generation and enhances angiotensinogen (Agt, the sole substrate of the renin-angiotensin system (RAS)) gene expression in rat renal proximal tubular cells (RPTCs).[34,35] The objective of this study was to define whether overexpressing CAT in RPTCs could attenuate ROS generation by high glucose and Ang II that lead to increased Agt and proapoptotic gene expression as well as to the induction of RPTC apoptosis in diabetic mice *in vivo*. For this purpose, we generated Tg mice overexpressing rat CAT (rCAT) in their RPTCs using the kidney-specific androgen-regulated protein (KAP)-gene promoter linked to rCAT cDNA. This promoter targets the gene of interest to proximal tubules, where the transgene will respond to androgen.[36]

RESULTS

Tissue-specific expression of the KAP2-rCAT transgene in Tg mice

Tg mice were generated to produce specific and inducible expression of rCAT in RPTCs using KAP2-rCAT construct (Figure 1a). Southern blot analysis confirmed the presence of

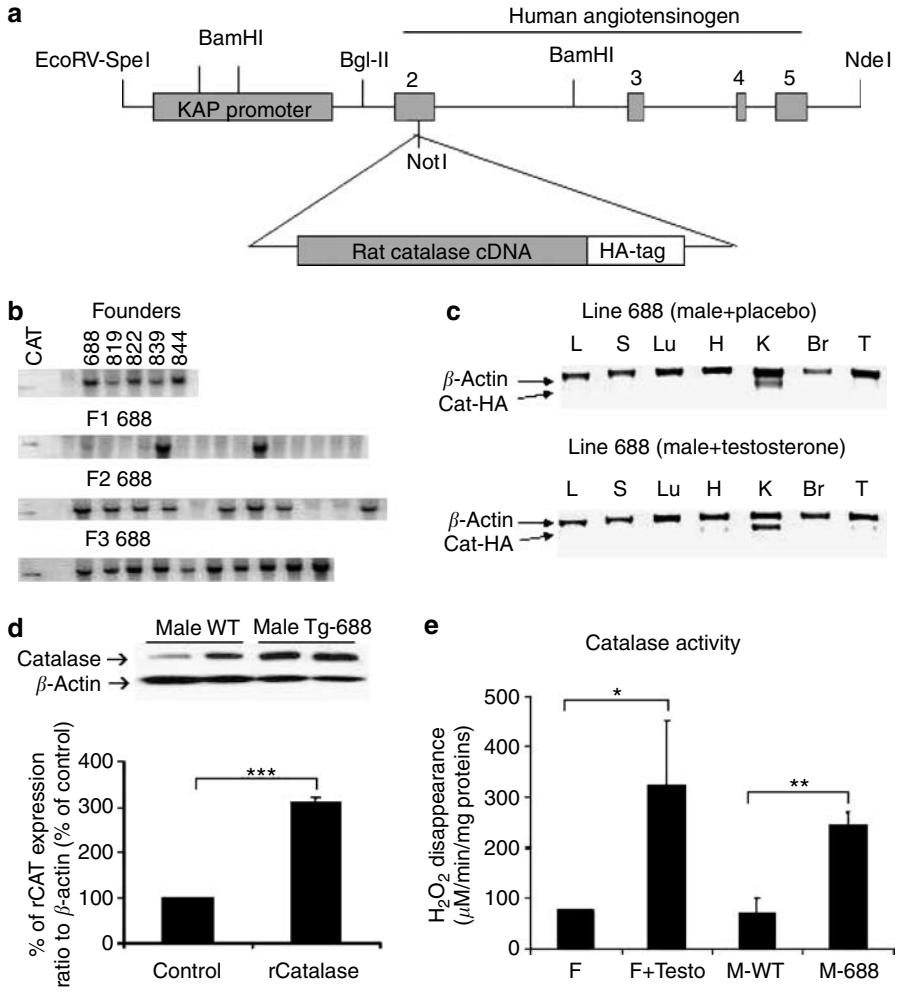

Figure 1 | Generation of Tg mice. (**a**) Schematic map of the KAP2rCAT construct. The rCAT cDNA fused with HA-tag was inserted into the *Not*I sites in exon II of human Agt gene. The transgene was excised as a *Spe*I and *Nde*I fragment for microinjection. (**b**) Southern blot analysis of tail biopsies of founders, F_1, F_2, and F_3 generations of Tg mice. Extracted genomic DNA was digested with *Bam*HI and migrated onto 0.8% agarose gel. The positive control (+) is composed of 10 pg of rCAT cDNA insert mixed with 10 μg of WT *Bam*HI-digested genomic DNA. (**c**) Southern blot of RT-PCR product showing tissue expression of rCAT-HA mRNA in male uninduced or induced with testosterone Tg mice. rCAT and β-actin fragments are indicated. Male (line no.; 688) mice were induced with placebo or testosterone for 2 weeks before RNA isolation. L, liver; S, spleen; Lu, lung; H, heart; K, kidney; Br, brain; T, testes. (**d**) Western blot analysis of rCAT expression in mouse RPT extracts of male WT and Tg mice (line no.;688). (**e**) CAT activity in mouse RPT extracts of female Tg mice with or without testosterone (Testo) induction and male (M) WT and Tg mice. CAT activity is defined as μmol of H_2O_2 consumed/min/mg of tissue protein at 10 mM H_2O_2 (*$P<0.05$, **$P<0.01$, ***$P<0.005$).

the transgene in heterozygote and homozygote animals (Figure 1b). Figure 1c displays the specific reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of rCAT-HA transgene expression in various tissues of Tg line 688. Male Tg mice express the transgene in the kidney but not in other tissues, and exogenous testosterone further enhances renal transgene expression.

An increase in CAT protein and CAT activity in the renal proximal tubules (RPTs) of male Tg mice without exogenous testosterone induction was also apparent in comparison with male wild type (WT) mice (Figure 1d and e). Testosterone administration evoked a three-fold increase in CAT activity in RPTs in female Tg mice (Figure 1e). Taken together, these results confirm that KAP2 directs rCAT transgene expression in RPTs of Tg mice. Thus, in subsequent experiments, we used male Tg mice without exogenous testosterone induction, as endogenous testosterone was sufficient to stimulate rCAT expression and activity in RPTs.

rCAT overexpression inhibits RPT ROS generation and ANG gene expression *ex vivo*

To demonstrate rCAT transgene functionality, freshly isolated RPTs were incubated *ex vivo* for 5 h in normal glucose medium (5 mM D-glucose plus 20 mM D-mannitol) in the absence or presence of Ang II (10^{-9} M) or in high glucose medium (25 mM D-glucose). ROS generation was augmented in RPTs of WT mice incubated in normal glucose medium plus (Ang II) or in high glucose medium (Figure 2a). No apparent increment of ROS generation, however, was observed in RPTs from Tg mice stimulated by Ang II or high-glucose. Similarly, Agt mRNA levels were also increased significantly in RPTs of WT mice incubated in high glucose or in normal glucose plus Ang II for 24 h (Figure 2b). The stimulatory effect of high glucose and Ang II on Agt mRNA expression was abolished in RPTs of Tg mice. Western blotting confirmed the effect of high glucose and Ang II on Agt protein expression in RPTs of WT and Tg mice (Figure 2c). These results indicate that ROS may mediate, at least in part, the effect of high glucose and Ang II on Agt mRNA and protein expression in RPTs *in vivo*.

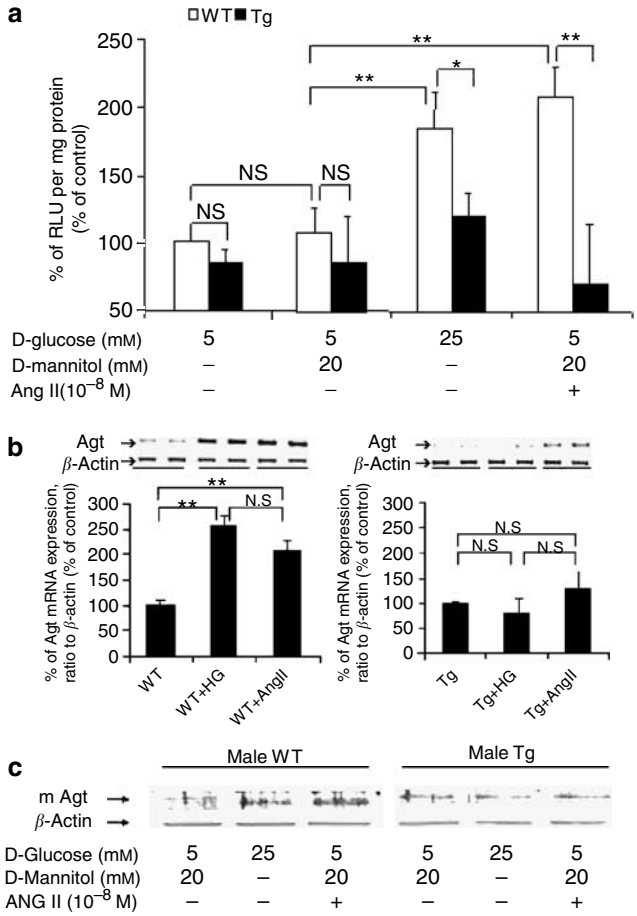

Figure 2 | Effect of high glucose or Ang II on ROS production and Agt mRNA and protein expression in mRPTs *ex vivo*. mRPTs from male WT or Tg mice were incubated in 5 mM D-glucose plus 20 mM D-mannitol (N.B.: D-mannitol was used to normalize the osmolarity as in 25 mM D-glucose medium) or 25 mM D-glucose serum-free Dulbecco's modified eagle's medium in the absence or presence of Ang II (10^{-9} M) for (**a**) 5 h or (**b**) 24 h. (**a**) ROS generation was assessed after 10 min incubation in Krebs buffer and expressed as RLU. (**b**) Agt and β-actin mRNA expression. The relative density of Agt mRNA bands was normalized with those of β-actin mRNA. Agt mRNA level in mRPTs incubated in 5 mM D-glucose Dulbecco's modified eagle's medium was considered as control (100%). Each point represents the mean ± s.d. of three independent experiments performed in duplicate (*$P < 0.05$, **$P < 0.01$; NS, not significant). (**c**) Western blotting of Agt and β-actin protein expression in mRPTs incubated in 5 mM D-glucose Dulbecco's modified eagle's medium with or with Ang II or in 25 mM D-glucose Dulbecco's modified eagle's medium.

Kidney hypertrophy and albuminuria in diabetic mice

Figure 3 presents the physical and biochemical data on both non-diabetic and diabetic WT and Tg mice. Diabetes in both WT and Tg mice as induced by streptozotocin (STZ) led to blood glucose elevation (Figure 3a), increases in 24-h urinary albumin excretion (Figure 3b), and higher kidney weight/body weight ratios (Figure 3c) compared with non-diabetic animals. Normalization of blood glucose by insulin reversed these parameters to non-diabetic levels.

Next, we investigated STZ-induced structural damage to the kidney by standard light microscopy. Glomeruli and RPTs appeared to be hypertrophic in both diabetic WT and Tg animals compared with their non-diabetic controls (Figure 4). Insulin treatment reversed the glomerular and RPT hypertrophy in diabetic WT and Tg mice. Furthermore, an increase of cellular edema and detachment and loss of RPTC brush-border were evident in diabetic WT kidneys. These pathological changes appeared to be attenuated in diabetic Tg kidneys. Insulin treatment ameliorated these pathological changes in both diabetic WT and Tg kidneys.

rCAT overexpression prevents increases in Agt and PAI-1 mRNA expression in diabetic RPTs

RT-quantitative PCR (RT-qPCR) analysis revealed that Agt mRNA expression is significantly increased (at least four- to five-fold) in RPTs of diabetic WT mice compared with non-diabetic WT mice (Figure 5a). Insulin significantly inhibited

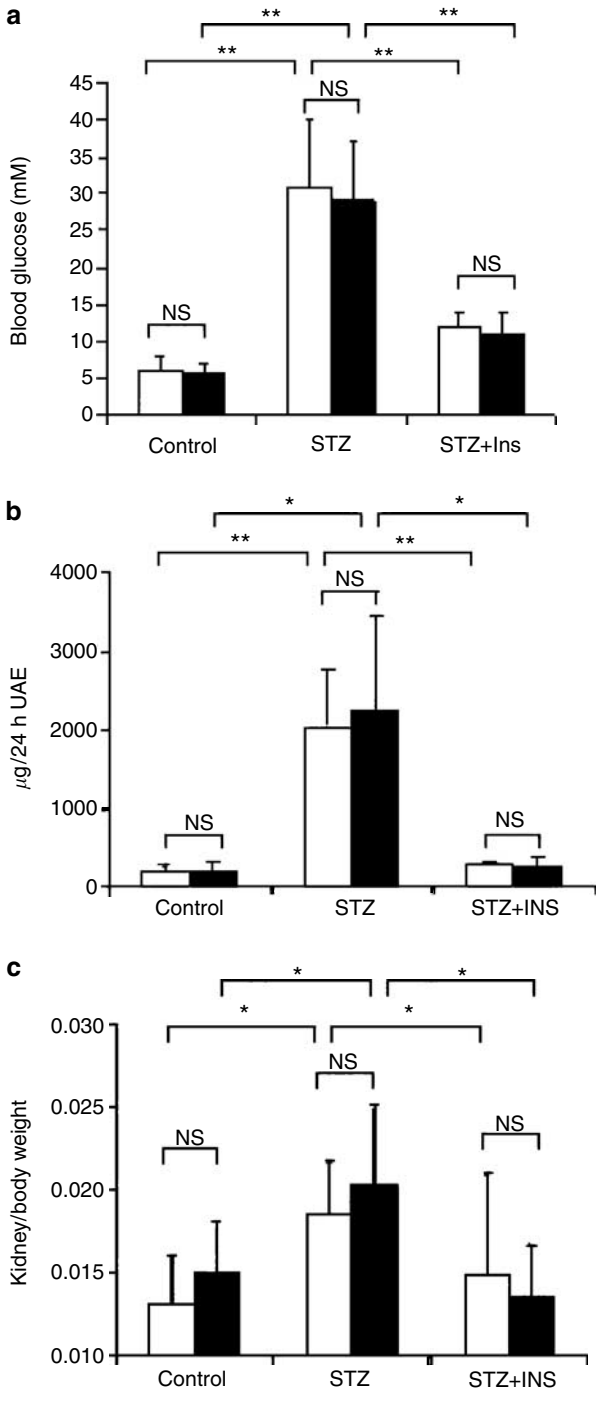

Figure 3 | Blood glucose, urinary albumin, and kidney/body weight ratio in male WT and Tg mice after 2 weeks of STZ-induced diabetes with or without normalization with insulin. (**a**) Blood glucose in WT (empty bars) and Tg (solid bars) mice. (**b**) twenty four-hour total urinary albumin of WT and Tg mice. (**c**) Kidney-to-body weight ratio. All data are expressed as means ± s.d., $n = 12$ (*$P < 0.05$, **$P < 0.01$; NS, not significant).

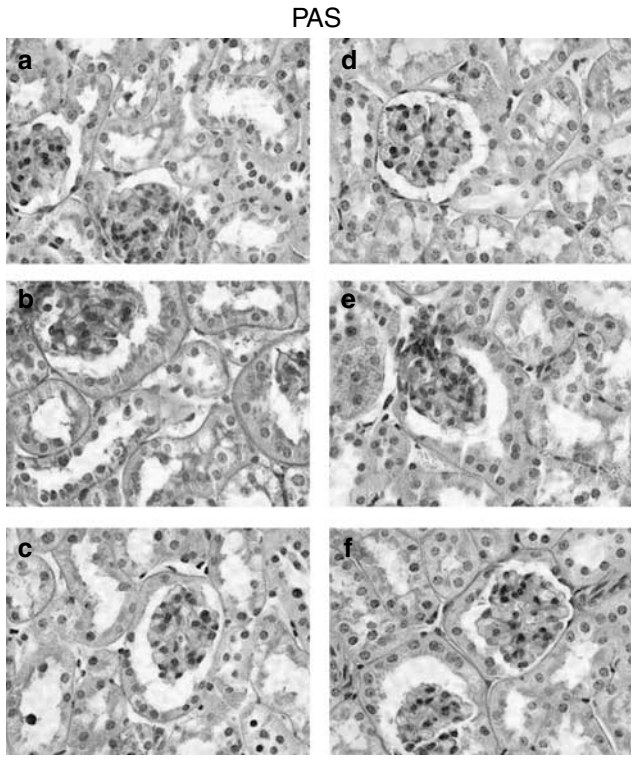

Figure 4 | PAS staining of male WT and Tg mouse kidneys 2 weeks after STZ-induction of diabetes with or without normalization with insulin. (**a**) Non-diabetic control littermate, (**b**) STZ-induced diabetic control littermate, (**c**) STZ-induced diabetic control littermate + insulin, (**d**) non-diabetic transgenic mouse, (**e**) STZ-induced diabetic transgenic animal, and (**f**) STZ-induced transgenic animal + insulin. Original magnification × 600.

RPT Agt mRNA expression in diabetic WT mice but could not normalize to levels as in non-diabetic WT mice. Similarly, Agt protein expression (immunostaining) was increased in diabetic WT mouse kidneys (Figure 5b–g). Insulin treatment attenuated but could not completely prevented RPT Agt expression in diabetic WT mice. In contrast, RPT Agt expression was not increased in diabetic Tg mouse kidneys with or without insulin treatment (Figure 5b–g). Non-immune normal rabbit serum displayed no immunostaining for ANG in both non-Tg and Tg mouse kidneys (data not shown).

Semiquantitative estimation of the number of Agt-positive stained tubules confirmed a three-fold increase of Agt-positive tubules in diabetic WT mice compared with non-diabetic WT mice (Figure 5h). Treatment with insulin attenuated but could not completely normalize the number of Agt-positive tubules to levels as in non-diabetics. In contrast, there was no apparent increase of Agt-positive stained tubules in diabetic Tg mice with or without insulin treatment compared with non-diabetic Tg mice.

Conventional RT-PCR analysis also showed that plasminogen activator inhibition-1 (PAI-1) and Agt mRNA (Figure 6a and b, left panel) levels in RPTs were significantly increased in diabetic WT mice compared with non-diabetic WT mice. rCAT overexpression prevented the increment of PAI-1 and Agt mRNA in RPTs of diabetic Tg mice (Figure 6a and b, right panel). Insulin treatment was not effective, however, in normalizing PAI-1 and Agt mRNA levels in diabetic WT mouse RPTs (Figure 6a and b, left panel). These studies confirm that hyperglycemia evokes ROS generation

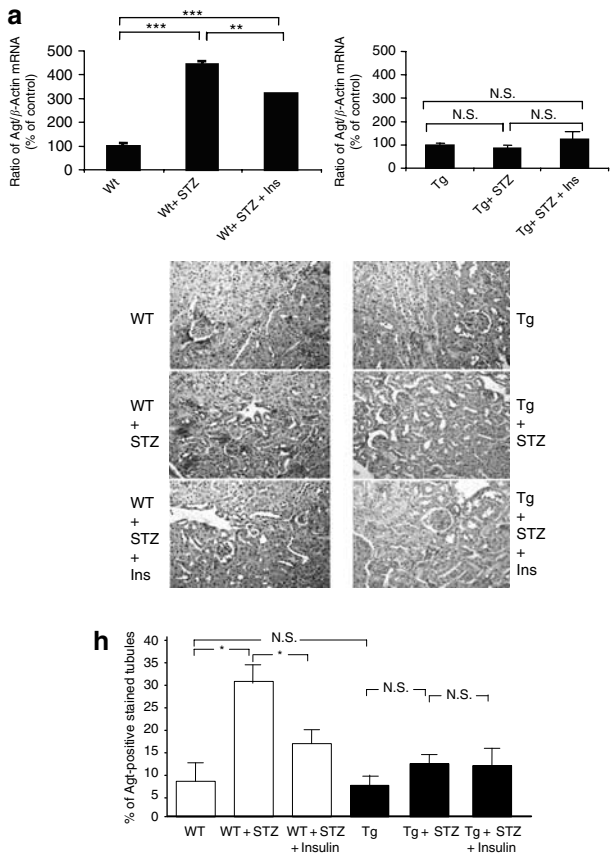

Figure 5 | RT-qPCR and immunostaining of respective mAgt mRNA and protein expression in RPTs of non-diabetic and disbetic WT and Tg mice with or without insulin supplementation. (**a**) After 2 weeks of diabetes, RPTs from WT and Tg mice were isolated and assayed for mAgt mRNA levels by RT-qPCR. The relative densities of mAgt mRNA were normalized with the β-actin mRNA. Agt mRNA levels in WT animals were considered as 100%. Each point represents the mean $\pm$ s.d. of 12 animals (**$P<0.01$, ***$P<0.005$). (**b**–**g**) Representative staining of Agt in male non-diabetic and diabetic WT and Tg mouse kidneys after 2 weeks of STZ-induced diabetes. Stain was performed using rabbit anti-rAgt polyclonal antibodies. (**b**) Non-diabetic control WT mouse kidney. (**c**) Diabetic control WT mouse kidney. (**d**) STZ-induced diabetic control WT mouse kidney + insulin. (**e**) Non-diabetic Tg mouse kidney. (**f**) STZ-induced diabetic Tg mouse kidney. (**g**) STZ-induced diabetic Tg mouse kidney + insulin. The arrow heads indicate the immunoreactive Agt in the tubules. Original magnification $\times 200$. (**h**) Bar graph showing quantitative analysis of Agt-positive stained tubules from male WT and Tg mouse kidneys after 2 weeks of STZ-induced diabetes with or without treatment with insulin. All data are expressed as means $\pm$ s.d., $n=6$ (*$P<0.05$, NS, not significant). WT (empty bars) and Tg (solid bars) mice.

(as implied by increased PAI-1 mRNA expression) and subsequently upregulates Agt mRNA expression in diabetic mice RPTs.

rCAT overexpression attenuates proapoptotic gene expression in diabetic RPTs

Figure 7 shows that the expression of p53 and Bax mRNA was significantly elevated in RPTs of diabetic WT compared with non-diabetic WT mice (Figure 7a and b, left panel). Increases in p53 and Bax mRNA levels were prevented in Tg mice overexpressing rCAT in their RPTs (Figure 7a and b, right panel). Insulin treatment was effective, however, in diminishing Bax mRNA but not p53 mRNA levels in diabetic WT mouse RPTs to controls.

To investigate whether the caspase-3 cascade was activated in diabetic RPTs, caspase-3 mRNA and activity were analyzed by RT-PCR and activity assay, respectively. Figure 8a reveals that caspase-3 mRNA expression was significantly increased in RPTs of diabetic WT compared with non-diabetic WT mice. Insulin treatment normalized the caspase-3 mRNA level. Furthermore, a significant increase in caspase-3 activity was observed in RPTs of diabetic WT mice (Figure 8b). In contrast, there were no apparent increases in caspase-3 mRNA levels and activity in diabetic Tg mice (Figure 8a and b).

rCAT overexpression prevents apoptosis in diabetic RPTs

Active caspase-3 and apoptosis in mouse kidneys were examined by immunohistochemistry and TUNEL assay, respectively. Active caspase-3 was present in RPTCs as well as in distal tubules (DT) of diabetic mice (Figure 9b) but not in non-diabetic WT and Tg mice (Figure 9a and c) and diabetic Tg mice (Figure 9d).

Furthermore, TUNEL assay revealed positively stained nuclei in RPTCs as well as in distal tubules of diabetic WT mice (Figure 10b and c) but not in RPTCs of non-diabetic WT and Tg mice (Figure 10a and d) and diabetic Tg mice (Figure 10e and f). These data demonstrate that apoptosis is clearly induced in RPTs and DT of diabetic mice and that CAT overexpression prevents RPTC apoptosis in diabetes.

DISCUSSION

We report here that rCAT overexpression in RPTs of Tg mice prevents ROS generation, Agt and proapoptotic gene expression as well as RPT apoptosis in diabetes, indicating occurrence of oxidative stress in mouse RPTs during early phases of diabetes. Consistently, upregulation of CAT reverses oxidative stress and RPT apoptosis *in vivo*.

To demonstrate that the antioxidant enzyme CAT can prevent ROS generation and attenuate kidney injury in diabetic mouse RPTs, Tg mice overexpressing rCAT specifically in their RPTs were generated. Tg line 688 showed that the transgene was highly expressed in male kidneys with or without exogenous testosterone induction but was not expressed in other tissues. Protein expression and activity of the transgene was confirmed by respective Western blotting and activity assay in isolated mouse RPTs. CAT activity was at least three- to four-fold higher in RPTs of Tg compared WT. Thus, these findings are consistent with our previous report as well as others that KAP2 directs the transgene expression in RPTCs.[36,37]

High glucose levels evoke ROS generation and enhance Agt gene expression in rat and mouse RPTC lines *in vitro*.[34,35,38,39] Consistently, high glucose and Ang II evoked ROS generation in RPTs of WT mice *ex vivo*. Most importantly, there was no apparent increase of ROS

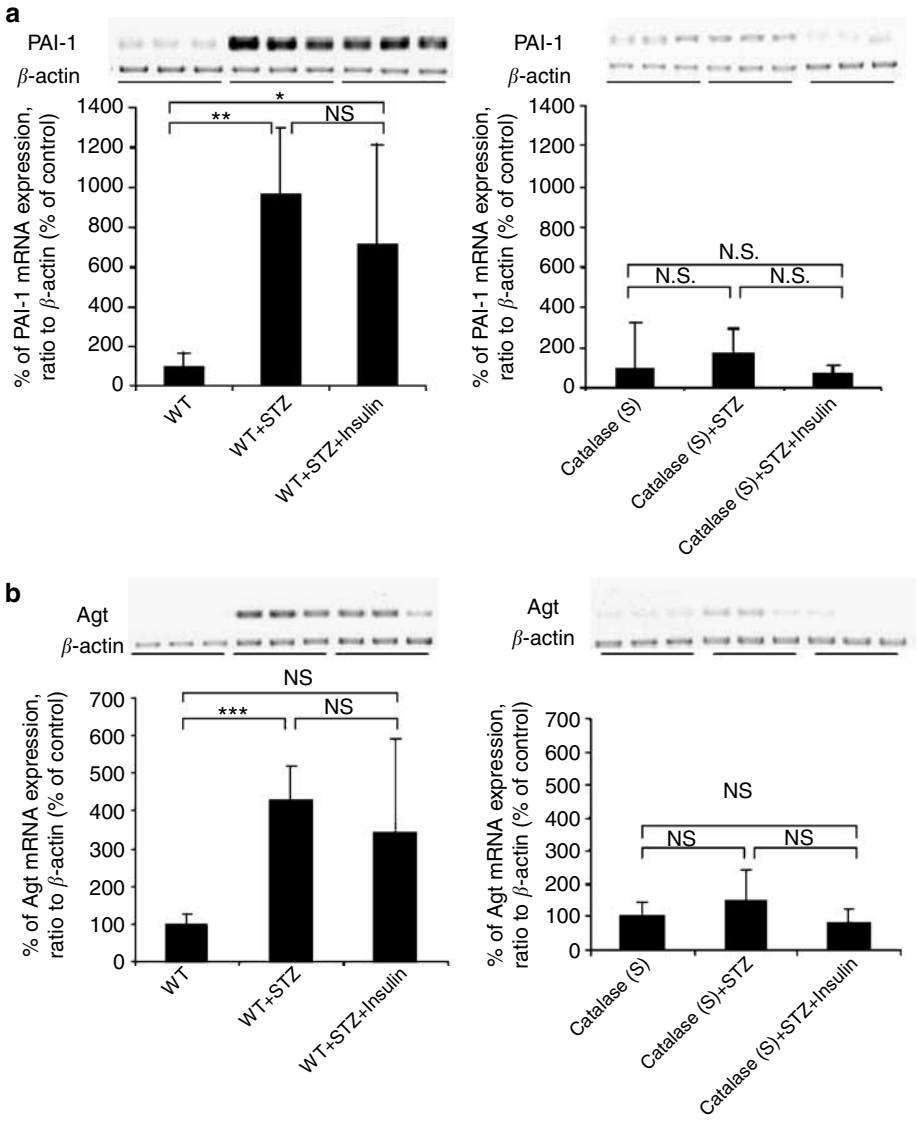

Figure 6 | RT-PCR assays of PAI-1 and Agt mRNA expression in RPTs of non-diabetic and diabetic WT and Tg mice with or without insulin supplementation. After 2 weeks of diabetes, RPTs from WT and Tg mice were isolated and assayed for (**a**) PAI-1 mRNA or (**b**) Agt mRNA levels by RT-PCR. The relative densities of PAI-1 and Agt mRNA were normalized with the β-actin mRNA. PAI-1 mRNA levels in WT animals were considered as 100%. Each point represents the mean $\pm$ s.d. of 12 animals (*$P<0.05$, **$P<0.01$, ***$P<0.005$; NS, not significant).

generation in RPTs of Tg male mice when stimulated with high glucose or Ang II. Furthermore, Agt mRNA levels were significantly elevated in WT RPTs incubated in high glucose or stimulated with Ang II, compared with RPTs incubated in normal glucose medium. rCAT overexpression completely abolished the increment of Agt mRNA in Tg RPTs stimulated by high glucose and Ang II. Western blotting confirmed the high glucose and Ang II upregulation of Agt protein expression in RPTs of WT but not Tg. Taken together, these observations suggest that rCAT overexpression in Tg RPTs is effective in preventing ROS generation stimulated by high glucose or Ang II and consequently abrogates Agt gene expression.

STZ is effective in inducing diabetes, with renal injury including renal hyperperfusion, albuminuria, glomerular and RPT hypertrophy, glomerular basement thickening and extracellular matrix protein gene expression in the mouse.[40,41] After 2 weeks of induction with STZ, all mice exhibited increased blood glucose, increased kidney to body weight ratio and proteinuria (a marker of kidney injury[42]). These parameters were not ameliorated by rCAT overexpression in RPTs of Tg mice, but these were completely abrogated by insulin treatment. Thus, our data indicate that rCAT overexpression in RPTCs alone was not sufficient to prevent the physiological changes seen in the diabetic kidney. We would suggest that the increased kidney/body weight ratio and albuminuria were because of the diabetic state *per se* and not to a nephrotoxic effect of STZ, as normalization of blood glucose by insulin reversed these parameters. Indeed, periodic acid schiff (PAS) staining of kidney sections from non-diabetic and diabetic mice (both WT and Tg mice) revealed structural damage to the kidney in STZ-treated

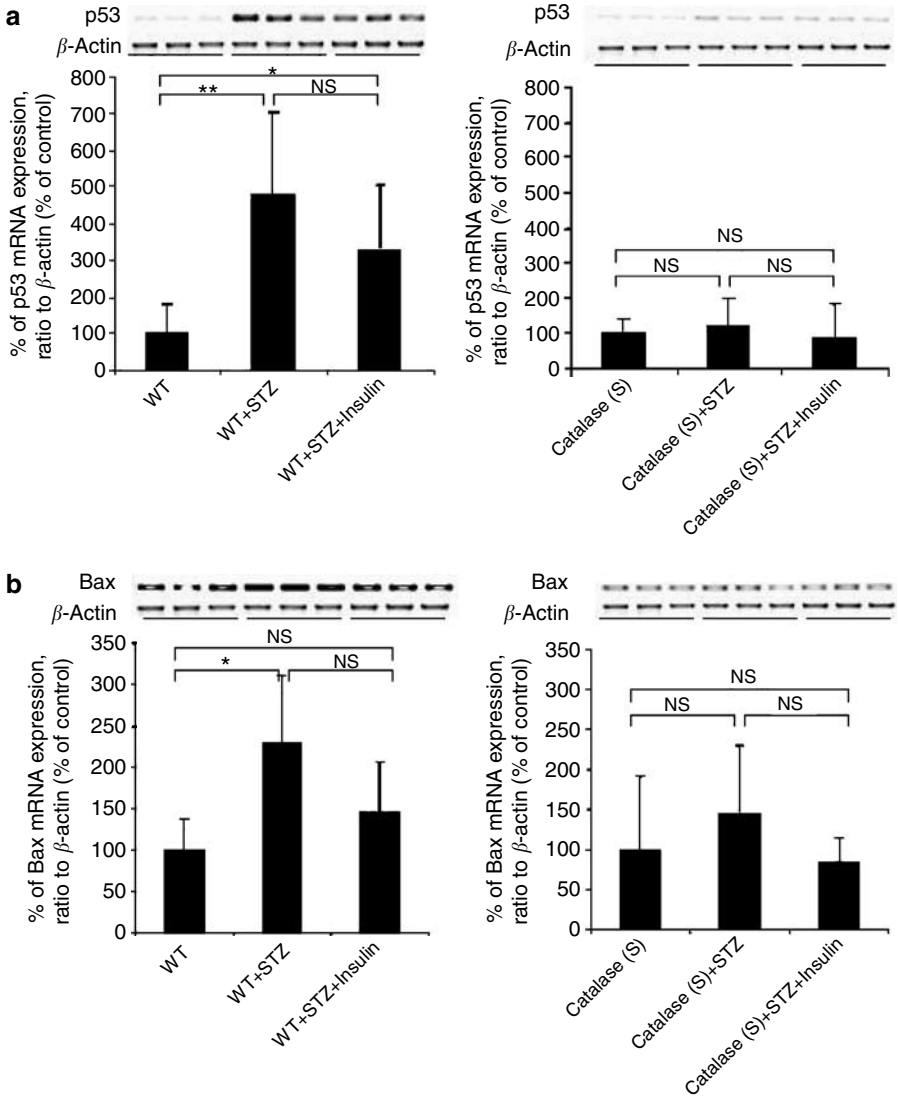

Figure 7 | Analysis of p53 and Bax mRNA expression in RPTs of non-diabetic and diabetic WT and Tg mice with or without insulin supplementation. After 2 weeks of diabetes, RPTs from WT and Tg mice were isolated and assayed for (**a**) p53 mRNA or (**b**) Bax mRNA levels by RT-PCR. The relative densities of p53 or Bax mRNA were normalized with the β-actin mRNA. p53 or Bax mRNA levels in WT animals were considered as 100%. Each point represents the mean ± s.d. of 12 animals (*$P<0.05$, **$P<0.01$; NS, not significant).

animals, including glomerular and tubular hypertrophy, loss of brush border, cellular edema and detachment. These pathological changes were ameliorated with insulin treatment in both WT and Tg kidneys.

PAI-1 is overexpressed in pathological conditions associated with renal fibrosis, including diabetic nephropathy.[43–45] ROS mediate high glucose-induced up-regulation of PAI-1 expression in cultured mesangial cells and diabetic glomeruli.[46,47] Thus, PAI-1 is a useful marker of ROS-inducible gene. PAI-1 and Agt mRNA expression were increased in RPTs of diabetic WT mice. Furthermore, the increase of PAI-1 and Agt mRNA was abolished in RPTs of male Tg mice, providing further evidence that ROS mediate the effect of high glucose-induced expression of Agt gene and RAS activation.

It is noteworthy that insulin treatment did not completely normalize Agt protein as well as PAI-1, ANG and p53 mRNA levels in diabetic WT mice. These data are consistent with our previous observations that prolonged exposure to high glucose evokes insulin resistance on inhibition of Agt mRNA expression in RPTCs,[35] and hyperglycemia induces insulin resistance on Agt gene expression in diabetic rat RPTCs.[48] Although the exact molecular mechanism(s) of high-glucose induction of insulin resistance remains uncertain, persistent ROS generation might be involved.[35]

High glucose is a potent inducer of apoptosis in RPTCs via ROS generation and activation of multiple caspases.[28–33] Consistent with these findings, we detected significantly enhanced expression of the proapoptotic gene p53, Bax, and caspase-3 mRNA in RPTs of WT diabetic mice, but not in RPTs of Tg diabetic mice, suggesting that ROS generation mediates, at least in part, by the induction of proapoptotic genes in WT diabetic mice. Active caspase-3 protein expression is found in RPTs and other renal cells (e.g., DT)

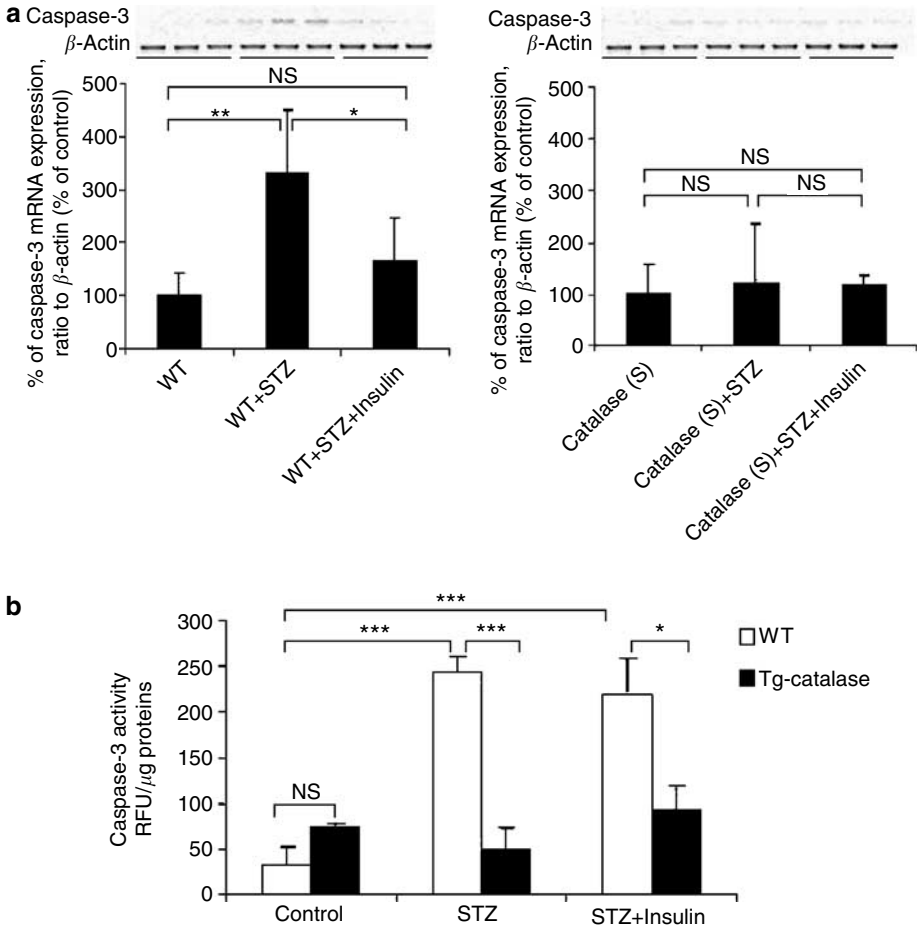

Figure 8 | Expression of capsase-3 mRNA and activity in RPTs of non-diabetic and diabetic WT and Tg mice with or without insulin supplementation. After 2 weeks of diabetes, RPTs from WT and Tg mice were isolated and assayed for (**a**) caspase-3 mRNA or (**b**) activity levels. (**a**) The relative densities of caspase-3 mRNA were normalized with β-actin mRNA control. Caspase-3 mRNA levels in WT animals were considered as 100%. Each point represents the mean $\pm$ s.d. of 12 animals (*$P<0.05$, **$P<0.01$; NS, not significant). (**b**) Similarly, caspase-3 activity levels in WT animals were considered as 100% . Each point represents the mean $\pm$ s.d. of 12 animals (***$P\leqslant0.001$. NS; not significant).

of diabetic WT mice, but not in RPTs of Tg diabetic mice. These observations hint that apoptosis is actually induced in the RPTCs of diabetic mice and that CAT overexpression may prevent it. Indeed, the TUNEL assay results revealed increases in the number of positively stained cells in the RPTs of WT diabetic mice compared with RPTs in non-diabetic WT mice. Additionally, the number of positively stained cells was significantly reduced in Tg diabetic mice.

In summary, our data indicate an important role for ROS in the pathogenesis of nephropathy in diabetes. CAT plays a relevant role in modulating diabetes-induced oxidative stress, proapoptotic gene expression, and apoptosis in RPTs *in vivo*. Selective activation of this enzyme may provide an alternative approach in preventing or reversing pathophysiological manifestations of diabetic nephropathy.

MATERIALS AND METHODS

Reagents

D(+)-glucose, D-mannitol, human Ang II, rabbit polyclonal antibodies against bovine CAT, and monoclonal antibodies against β-actin were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). A rabbit polyclonal antibody against rat Agt was generated in our lab (JSDC) and reported previously.[49] The anti-rAgt antibody cross-reacts with rat and mouse Agt but has no cross-reactivity with murine pituitary hormones and albumin as well as plasma proteins from dog, cat, hamster or human.[49] rCAT cDNA was a gift from Dr Paul E Epstein (University of Louisville, Louisville, KY, USA). The pKAP2 plasmid containing the KAP promoter responsive to testosterone stimulation was obtained from Dr Curt D Sigmund (University of Iowa, Iowa, IA, USA) and has been described elsewhere.[36] Placebo pellets or pellets containing 5 mg testosterone with a 21-day release schedule (Cat. no. A-121) were purchased from Innovative Research of America (Sarasota, FL, USA). Oligonucleotides were synthesized by InVitrogen Inc. (Burlington, ON, Canada). Restriction and modifying enzymes were purchased from either InVitrogen Inc., La Roche Biochemicals (Dorval, QC, Canada) or Amersham-Pharmacia Biotech Inc. (Baie d'Urfé, QC, Canada).

Generation of KAP2-rCAT Tg mice

Tg mice overexpressing rCAT in their RPTCs were generated according to the procedures we described previously for Tg mice overexpressing rAgt in RPTCs.[37] Briefly, cDNA encoding full-length

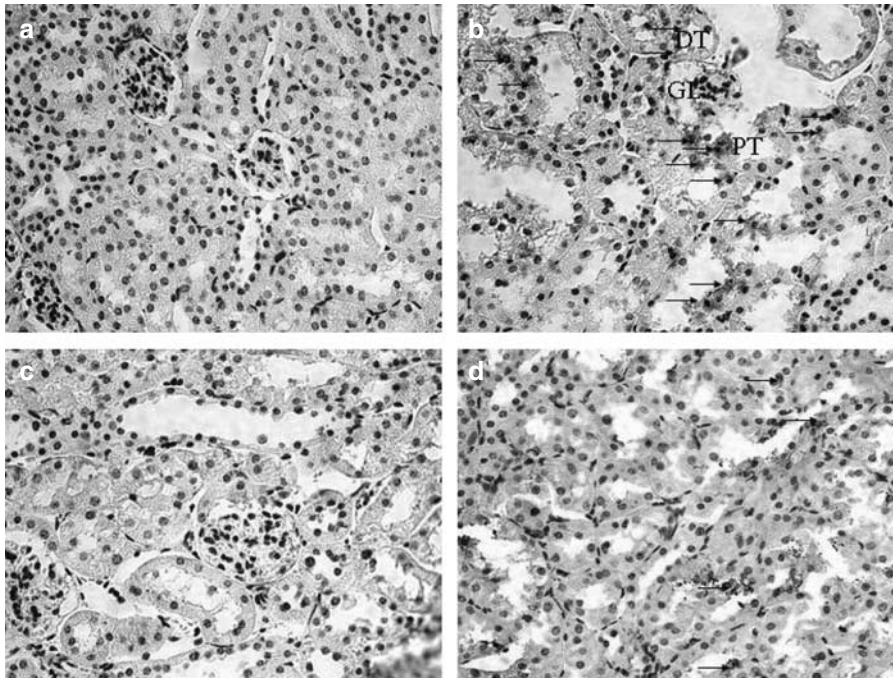

Figure 9 | Immunohistochemical staining of α-active caspase-3 in male non-diabetic and diabetic WT and Tg mouse kidneys after 2 weeks of STZ induction by employing rabbit anti-α-active caspase-3 polyclonal antibodies. (**a**) Non-diabetic control WT mouse kidney. (**b**) Diabetic control WT mouse kidney. (**c**) Non-diabetic Tg mouse kidney. (**d**) Diabetic Tg mouse kidney. The arrows indicated the immunoreactive α-active caspase-3. Original magnification × 200.

rCAT fused with HA-tag and terminating codon at the 3′ end (NotI site flanked at both 5′- and 3′-terminali) was inserted into pKAP2 plasmid at the NotI site of exon II of human angiotensinogen (hAgt) gene. The isolated KAP2-rCAT transgene was then microinjected into one-cell fertilized mouse embryos using a standard procedure.[37] Five founder mice were identified. The positive Tg founders were then crossed with WT C57Bl6 mice for F1 generation. Breeding was continued until homozygous F3 and F4 Tg mice were obtained.

The Tg mice used in our experiments were males, aged 10–14 weeks at the time of data collection, unless otherwise noted. Non-Tg (WT) littermates served as controls. All animals received standard mouse chow and water *ad libitum*. Animal care and procedures were approved by the Animal Care Committee of the CHUM.

Analysis of transgene expression

Placebo pellets or pellets containing 5 mg testosterone with a 21-day release schedule were implanted surgically in WT and Tg mice in order to see whether exogenous testosterone further increased expression in males and to induce it in females.[37] Two weeks after testosterone implantation, the animals were killed and various tissues were harvested and snap-frozen on dry ice. Total RNA was isolated from various tissues and subjected to RT-PCR analysis.[37] The rCAT sense primer[50] and the HA antisense primer (Table 1), β-actin forward primer and reverse primer[51] (Table 1) were used for PCR. rCAT-HA mRNA levels were normalized by corresponding β-actin mRNA levels.

Morphological studies and immunohistochemical staining

Kidneys were collected in Tissue-Tek cassettes (VWR Canlab, Montreal, QC, Canada), dipped immediately in ice-cold formaldehyde (10% in phosphate-buffered saline), and fixed for 24 h at 4°C. The cassettes were then processed by the CHUM Pathology Department. Tissue sections (4–5 specimen per group) were stained with PAS and analyzed visually under a light microscope by an observer unaware of the treatments.

Immunohistochemical staining was performed via a standard avidin–biotin–peroxidase complex method (ABC Staining System, Santa Cruz Biotechnologies, Santa Cruz, CA, USA).[37] Renal sections were incubated with non-immune serum (1:100 dilution) or primary anti-rAgt (1:100 dilution)[49] or anti-CAT polyclonal antibody (1:500 dilution) or anti-active alpha (α) caspase-3 polyclonal antibody (Chemicon, Temecula, CA, USA) (1:50 dilution) for 1 h at room temperature. Then, a biotinylated secondary antibody was added, followed by the addition of preformed ABC reagent supplied by kit. Agt, CAT, and α-active caspase-3 proteins were visualized by color development with 3, 3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

The percentage of tubules that stained positive for Agt in kidneys of WT and Tg mice was estimated semiquantitatively. Briefly, 6–10 fields per animal were randomly selected from group of animals. Then the total number of tubules containing Agt-stained cells was divided from the total number of tubules counted and multiplied by 100 to derive the percentage of Agt-positive stained tubules.

Induction of diabetes

Male WT and Tg mice at 10–12 weeks of age each were divided into three subgroups (12 mice per group): (1) vehicle-injected control group. These animals were injected intraperitoneally (i.p.) with 10 mM sodium citrate buffer, pH 4.0, in 0.9% saline. (2) STZ-induced diabetic group. After overnight fasting, these animals were administered 150 mg/kg of STZ dissolved in 10 mM sodium citrate buffer, pH 4.0, in 0.9% saline, i.p. Forty-eight hours after STZ

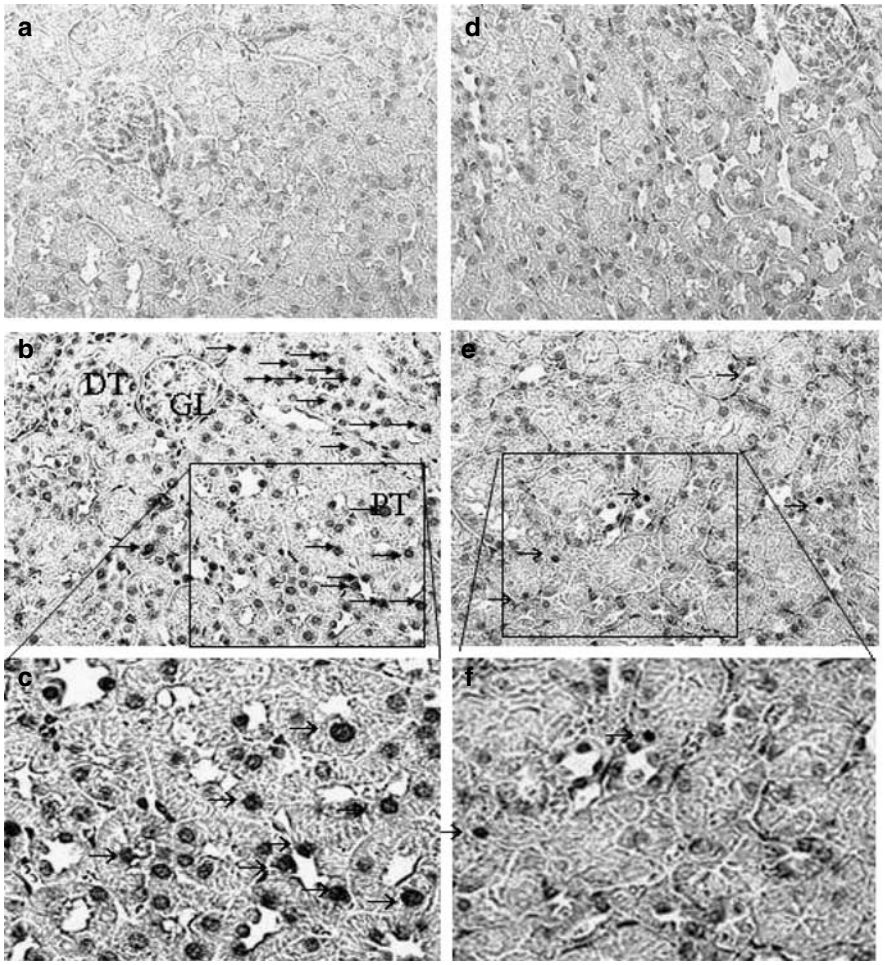

Figure 10 | Apoptosis in male non-diabetic and diabetic WT and Tg mouse kidneys after 2 weeks of STZ induction analyzed by terminal transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling staining. (**a**) Non-diabetic control WT mouse kidney, original magnification $\times 10$. (**b**) Diabetic control WT mouse kidney, magnification $\times 10$. (**c**) Diabetic control WT mouse kidney, original magnification $\times 40$. (**d**) Non-diabetic Tg mouse kidney, original magnification $\times 10$. (**e**) Diabetic Tg mouse kidney, magnification $\times 10$. (**f**) Diabetic Tg mouse kidney, original magnification $\times 400$. The arrows indicated the apoptotic cells. GL, glomerulus; PT, proximal tubule; DT, distal tubule. Original magnification $\times 200$.

administration, blood was assayed for glucose levels with a Side-Kick Glucose Analyzer (Model 1500, Interscience, ON, Canada). Only mice with blood glucose >400 mg/dl or >20 mM were included in the study. (3) Insulin-treated diabetic group. Confirmed STZ-induced diabetic mice received a subcutaneous insulin implant, Linplant (Linshin, Scarborough, ON, Canada) at 24 h after STZ administration to maintain euglycemia. After 2 weeks of diabetes with or without insulin implant, the animals were killed with CO_2. Twenty-four hours before killing, animals were housed individually in metabolic cages. Urine samples were collected and assayed for glucose and ketone levels (Keto-Diastix, Bayer Inc., Healthcare Division, Toronto, ON, Canada) and albuminuria (ELISA, Albuwell, Exocell, Philadelphia, PA, USA). Kidneys were removed immediately after killing. Kidneys from the left side were sectioned in half longitudinally and one-half was taken for pathology studies. The remaining half of the left kidney and the right kidney from each animal were pooled for proximal tubule isolation by Percoll gradient with slight modifications.[48,38]

Aliquots of freshly isolated mRPTs from individual mice were used immediately for protein extraction or total RNA isolation or assay of CAT and caspase-3 activity. The remaining mRPTs were then pooled, suspended in serum-free culture medium,[38] incubated at 37°C in the absence or presence of Ang II (10^{-9} M) or in 25 mM D-glucose medium for 16 h. At the end of the incubation period, mRPTs were harvested and assayed for ROS generation and gene expression.

Western blot analysis

Western blot analysis was performed according to the method described previously.[37,48,38] The membrane was first blotted with rabbit anti-Agt or anti-CAT, and then reblotted with anti-β-actin monoclonal antibodies and chemiluminescent developing reagent.

CAT activity assay

CAT activity in mRPTs was determined after decomposition of 10 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0, by monitoring the linear decrease of absorbance at 240 nm for 30 s as described by Aebi.[52] The amounts of H_2O_2 decomposed by CAT were calculated with the millimolar extinction coefficient of H_2O_2 ($\epsilon_{240} = 0.0436$). CAT activity was defined as μmol of H_2O_2 consumed/min/mg of tissue protein at 10 mM H_2O_2.

Table 1 | Primers for conventional RT-PCR

Agt S N+676 to N+695 (5′-CCT CGC TCT CTG GAC TTA TC-3′)
Agt AS N+882 to N+901 (5′-CAG ACA CTG AGG TGC TGT TG-3′)
(BC087679)
Bax S N+57 to N+76 (5′-TCA TGA AGA CAG GGG CCT TTT-3′)
Bax AS N+293 to N+273 (5′-CAA TCA TCC TCT GCA GCT CCA-3′)
(L22472)
Caspase-3 S N+548 to N+567 (5′-CAG GCC TGC CGG GGT ACG GA-3′)
Caspase-3 AS N+897 to N+878 (5′-TGA TAA AAG TAC AGT TCT TT-3′)
(Y13086)
Catalase S2 N+1152 to N+1170 (5′-CAC TCA CCG CCA CCG CCT G-3′)
HA-Catalase AS N+1582 to N+1567 (5′-AAG CGG CCG CTT AGG CGT AGT CAG GCA CGT CGT AAG GAT ACA GGT TAG CTT TTC-3′)
HA AS (5′-GGC GTA GTC AGG CAC GTC GT-3′)
(BC081853)
PAI-1 S N+972 to N+989 (5′-CTC ATC AGA CAA TGG AAG AGC-3′)
PAI-1 AS N+1200 to N+1180 (5′-CAT CAC TTG CCC CAT GAA GAG-3′)
(M33960)
P53 S N+1 to N+19 (5′-AAA GCG GCC GCA TGG AGG ATT CAC AGT CGG 3′)
P53 AS N+1272 to N+1254 (5′-AAA GCG GCC GCA GTC TGA GTC AGG CCC CAC-3′)
(AB020317)
β-Actin S N+155 to N+179 (5′-ATG CCA TCC TGC GTC TGG ACC TGG C-3′)
β-Actin AS N+115 to N+139 (5′-AGC ATT TGC GGT GCA CGA TGG AGG G-3′) (NM 031144)

Agt, angiotensinogen; AS, antisense; PAI-1, plasminogen activator inhibitor-1; S, sense.

ROS generation

ROS production was monitored by the lucigenin method with minor modifications.[34,38] ROS generated in mRPTs were normalized with protein concentration and expressed as relative light units per mg protein.

Conventional RT-PCR and real time RT-qPCR assays for gene expression

Total RNA was used in conventional RT-PCR to quantify the amount of PAI-1, Agt, p53, Bax and caspase-3 mRNA expressed in mRPTs according to previously described protocols.[34,35,38] The forward and reverse primers corresponding to PAI-1,[53] Agt,[54] p53,[55] Bax,[56] caspase-3,[57] and β-actin cDNA[51] in the RT-PCR assays are described in Table 1. PAI-1, Agt, p53, Bax, and caspase-3 mRNA levels were normalized by corresponding β-actin mRNA levels.

The total RNA was also used for RT-qPCR for mouse Agt as described previously.[58] The primers for mouse Agt (NM_007428): sense 5′-ACA GAC ACC GAG ATG CTG TT-3′ and antisense 5′-CCA CGC TCT CTG GAT TTA TC-3′ were used in RT-qPCR.

Caspase-3 activity assay

Caspase-3 activity was assayed on frozen (−80°C) mPTs using caspase-3 assay kits (BD Bioscience Pharmingen, Mississauga, ON, Canada).

Terminal transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling assay

Kidneys from WT and Tg mice were removed immediately after killing. Formaldehyde (10%)-fixed, paraffin-embedded kidney sections (5-μm thick) were deparaffinized in xylene and rehydrated. Apoptosis was quantified with a TUNEL kit (La Roche Biochemicals) according to the supplier's instructions.

Statistical analysis

Data were expressed as means ± s.d. Student's *t*-test was used initially to analyze the statistical significance between experimental groups. Then, the data were analyzed by one-way analysis of variance and the Bonferroni test. $P < 0.05$ values were considered to be statistically significant.

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