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Hyperglycemia, p53 and mitochondrial pathway of apoptosis are involved in the susceptibility of diabetic models to ischemic acute kidney injury

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Abstract

Patients with chronic kidney diseases, including diabetic nephropathy, are more susceptible to acute kidney injury (AKI) and have a worse prognosis following AKI. However, the underlying mechanism is unclear. Here we tested whether diabetic mice were more sensitive to AKI and show that renal ischemia-reperfusion induced significantly more severe AKI and higher mortality in the streptozotocin and the Akita diabetic mouse models. The severity of AKI in the mice correlated with their blood glucose levels. *In vitro*, high glucose-conditioned renal proximal tubular cells showed higher apoptosis and caspase activation following ATP-depletion and hypoxic injury, accompanied by a heightened mitochondrial accumulation of Bax and release of cytochrome c. In response to injury, both glucose-conditioned renal proximal tubular cells and diabetic kidney tissues showed markedly higher p53 induction. Suppression of p53 diminished the sensitivity of high glucose-conditioned cells to acute injury *in vitro*. Moreover, blockade of p53 by pifithrin- α , siRNA, or proximal tubule-targeted gene ablation reduced ischemic AKI in diabetic mice. Insulin reduced blood glucose in diabetic mice and largely attenuated their AKI sensitivity. Thus, our results suggest the involvement of hyperglycemia, p53 and mitochondrial pathway of apoptosis in the susceptibility of diabetic models to AKI.

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Keywords

acute kidney injury; diabetic nephropathy; high glucose; ischemia-reperfusion; mitochondria; p53

Introduction

Chronic kidney disease (CKD) is a condition of progressive loss of renal function in months to years. As a common disease, CKD affects 10% or more of adults in various countries including the United States (1). The major causes of CKD include diabetes mellitus (DM), hypertension, and glomerulonephritis. In developed countries, DM is the leading cause of CKD, which accounts for almost half of the cases of end-stage kidney diseases (2). DM-associated kidney disease, also known as diabetic nephropathy (DN), is characterized pathologically by abnormalities in glomerular, tubulo-interstitial, and vascular compartments, including extracellular matrix accumulation, thickening of basement membrane, cellular hypertrophy, and apoptosis (3). Hyperglycemia or high blood glucose is a key factor driving the functional and pathological changes in kidneys in DN. In kidney cells, high glucose induces a plethora of stress responses, including metabolic shift, mitochondrial dysfunction, endoplasmic reticulum stress, and oxidative stress, just to name a few (3, 4). Together, these cellular events lead to a series of pathological changes in kidney tissues, culminating in the gradual or progressive loss of renal function.

Acute kidney injury (AKI) is a disease of rapid loss of renal function. Traditionally, CKD and AKI are classified as two separate kidney disorders. However, recent epidemiologic and basic science research has revealed an important, bidirectional relationship between them (5-7). On one hand, AKI may contribute to the development and progression of CKD. On the other hand, CKD is a major risk factor for AKI. As such, CKD patients are predisposed to AKI and AKI leads to a much worse prognosis in CKD patients (including those with DM) than non-CKD patients (8, 9). Recent research has gained significant insights into the mechanism underlying the progression of AKI to CKD, involving maladaptive repair in renal tubules, vascular rarefaction, fibrosis and interstitial inflammation (10-12). However, the AKI sensitivity in CKD has received much less attention. Nonetheless, several studies reported the AKI sensitivity or susceptibility of diabetic animals (13-17). Mechanistically, Kelly et. al. suggested the involvement of inflammation (17), while Gao et. al. further demonstrated that the AKI sensitivity of diabetic mice can be suppressed by a TNF- α neutralizing antibody, supporting a role of TNF- α -related inflammatory response (13).

The current study has investigated ischemic AKI in diabetic models. We show that renal ischemia-reperfusion induces more severe AKI in diabetic mice than non-diabetic mice. The severity of AKI in these mice correlates with their blood glucose levels. Mechanistically, both glucose-conditioned cells and diabetic kidney tissues are sensitized to mitochondrial pathway of apoptosis. Moreover, in response to injury p53 is induced markedly in these cells and tissues. Suppression of p53 diminishes the sensitivity of high glucose-conditioned cells and diabetic kidney tissues to acute injury, revealing a role of p53 in their injury susceptibility.

Results

Sensitivity of STZ-induced diabetic mice to ischemic AKI

AKI leads to a significantly worse outcome in diabetic patients, as indicated by the rates of mortality and progression to end stage renal disease (8, 9). To recapitulate the observation in animal models, we initially compared ischemic AKI in STZ-induced diabetic mice (DM) and non-diabetic mice without STZ treatment (ND). Functionally, 20 minutes of bilateral renal ischemia followed by 24, 48 and 72 hours of reperfusion (I/R24, I/R48 and I/R72) resulted in marked increases in BUN in both ND and DM mice, but the BUN increases were significantly higher in DM mice than ND mice (Figure 1A). Consistently, I/R48 and I/R72 resulted in significantly higher serum creatinine in DM mice than that in ND mice (Figure 1B).

Corroborating with the functional analysis, renal histology revealed significantly more tissue damage in DM mice after ischemic injury. I/R72 led to severe tubular damage, lysis and necrosis in renal cortex and outer stripe of outer medulla in DM mice, whereas in ND mice, much fewer tubules were injured and the injury in individual tubules was also less severe mainly showing tubular dilation and loss of brush boarder (Figure 1C). Quantitatively, cortical and outer medulla tubular damage after renal ischemia-reperfusion was marginally higher at I/R24 and I/R48, but became significantly higher at I/R72 in DM mice than ND mice (Figure 1D). Moreover, apoptotic cells revealed by TUNEL assay were rare in sham control kidney tissues of both ND and DM mice (Supplementary Figure 1). After I/R, the number of apoptotic cells increased to 82/mm² cortical tissue in DM mice, but only 41/mm² cortical tissue in ND mice (Figure 2F; Supplementary Figure 1).

Finally, we recorded animal death after 20 and 22 minutes of bilateral renal ischemia (Figure 1F). All non-diabetic mice survived during the observation period of 14 days. In STZ-diabetic mice, there was a gradual loss of animals between day 2 and 4 of reperfusion and as expected, 22 minutes of ischemia induced higher animal death than 20 minutes of ischemia. Kaplan-Meier analysis indicated ischemic AKI led to significantly higher death in STZ-diabetic mice than that in non-diabetic mice.

Akita diabetic mice are susceptible to ischemic AKI

Akita mice have a single point mutation in the insulin 2 gene that causes misfolding of the insulin protein triggering hyperglycemia or diabetes from 3-4 weeks of age (18). We used Akita mice to further examine the AKI sensitivity in diabetes (Figure 2). Compared to sham-operated control, 23 minutes of bilateral renal ischemia followed by 24 and 48 hours of reperfusion (I/R24, I/R48) resulted in modest increases in BUN in wild-type (Ins2^{+/+}) mice; however in Akita diabetic (Ins2^{Akita/+}) mice, BUN increased dramatically after I/R24 and I/R48 (Figure 2A). Consistently, serum creatinine increased to much higher levels in Akita diabetic mice after I/R48 than wild-type littermates (Figure 2B).

In histology, I/R48 led to severe tubular damage in Akita diabetic mice, showing extensive tubular dilation and distortion, loss of brush border, cell lysis, and sloughed debris, while in wide type mice, considerably fewer tubules were injured and the injury in individual tubules was also less severe (Figure 2C). By quantification, tubular damage in renal cortex and outer

medulla after I/R was significantly higher in Akita diabetic mice (Figure 2D). In addition, I/R induced significantly higher apoptosis in Akita diabetic kidneys than that in wild type tissues (Figure 2E, 2F). Renal I/R also induced severe hemorrhage in the inner medulla in Akita mice, but not in wild-type littermates (data not shown). Together, the observations from both STZ-induced and Akita models indicate the hypersensitivity of diabetic mice to ischemic AKI.

Correlation between BUN and fasting glucose levels

Hyperglycemia is a central initiating factor in the pathogenesis of diabetic complications, including DN. We asked whether it contributes to the AKI sensitivity in diabetic models. To address this, we analyzed the correlation between the levels of fasting blood glucose and BUN in the samples collected from the STZ and Akita models. As shown in Figure 3, BUN measured after ischemic AKI correlated well with the blood glucose level in mice with an R value of 0.85, suggesting that hyperglycemia may contribute to the AKI sensitivity in diabetes.

High glucose-conditioned RPTC cells are sensitized to apoptosis following ATP-depletion and hypoxia

To study the mechanism of the AKI sensitivity of diabetic/hyperglycemic kidney tissues, we established an in vitro model using high glucose-conditioned renal proximal tubular cells (RPTC). To this end, RPTC cells were cultured for two weeks in a medium containing 30 mM glucose (high glucose), while the control groups were grown with 5.5 mM glucose (normal glucose) or 5.5 mM glucose with 24.5 mM mannitol. These cells were then subjected to azide treatment for ATP-depletion or severe hypoxia (to model in vivo ischemia), followed by recovery in full culture media (to model in vivo reperfusion). After the treatment, many cells developed typical apoptotic morphology, showing cellular and nuclear condensation and fragmentation. By cell counting, hypoxic treatment induced ~20% apoptosis in normal glucose or mannitol culture cells, but 60% in high glucose-conditioned cells (Figure 4A; Supplementary Figure 2). Consistently, high glucose-conditioned cells had significantly higher caspase activity than other two groups (Figure 4B). Similar observations were shown for the treatment of azide-induced ATP depletion (Figure 4C, 4D).

Increased Bax translocation and cytochrome c release in high glucose-conditioned RPTC cells and Akita diabetic mice during acute injury

Hypoxia in vitro and renal ischemia in vivo activate the intrinsic pathway of apoptosis in kidney tubular cells (19-22), which is characterized by Bax accumulation in mitochondria and permeabilization of mitochondrial outer membrane for release of apoptogenic factors, such as cytochrome c (cyt. c). To analyze these apoptotic events, cells were fractionated into cytosolic fraction and the mitochondrial-enriched organellar fraction for immunoblot analysis of Bax and cytochrome c. In control cells, Bax was mainly detected in the cytosolic fraction (Figure 5A: lanes 1, 5, 9). During hypoxia, Bax gradually translocated to the mitochondria-enriched organellar fraction; notably, high glucose-conditioned cells showed an earlier and higher Bax translocation (Figure 5A: lanes 6-8 vs. 2-4 or 10-12). Concomitantly, high glucose-conditioned cells showed an earlier and higher cytochrome c release during hypoxic treatment (Figure 5A).

In kidney tissues, I/R induced a moderate Bax accumulation in mitochondria in wild-type mice (Figure 5B: lane 2 vs. 1). Bax accumulation in mitochondria was more evident in Akita diabetic mice following I/R (Figure 5B: lane 3 vs. 2). In line with these Bax results, cytochrome c release into cytosol appeared higher in Akita diabetic kidneys than wild-type tissues (Figure 5B: lane 3 vs. 2).

Heightened p53 induction in high glucose-conditioned RPTC cells and Akita diabetic mice

The results described above support the involvement of the intrinsic pathway of apoptosis in the AKI sensitivity of diabetic tissues. How does high glucose or diabetes enhance the intrinsic pathway that is centered on mitochondria? In 2003, Kelly and colleagues reported p53 induction during renal ischemia-reperfusion, which interestingly showed a main localization at mitochondria in renal tubular cells.(23) The role of p53 in ischemic AKI was established by inhibitory studies using pharmacological inhibitors and siRNA and very recently by using kidney tubule-specific p53-knockout mouse models.(23-25) We therefore postulated a role of p53 in the AKI sensitivity of diabetic or hyperglycemic cells and tissues. In RPTC cells, p53 was marginally (~1.5 fold over control by densitometry analysis) up-regulated and phosphorylated at serine-15 after high glucose conditioning (Figure 6A: lane 2 vs. 1 and 3). Upon azide-induced injury, high glucose-conditioned cells showed ~2 fold higher p53 induction than normal glucose cells (Figure 6B: lane 4 vs. 3). In kidney tissues, some (but not all) Akita diabetic mice showed a higher basal level of p53 than wild-type tissues (Supplementary Figure 3). After renal ischemia-reperfusion, diabetic kidney tissues showed a consistent p53 up-regulation, which was ~2 fold higher than that of non-diabetic, wild-type mice (Figure 6C: lanes 6-8 vs. 3-5). Similarly, renal ischemia-reperfusion induced higher p53 in the kidney tissues from STZ-treated diabetic mice (Figure 6D: lanes 3-5 vs. 6-8).

Inhibition of p53 reduces ATP-depletion-induced apoptosis in high glucose-conditioned cells

Is p53 a key factor in the AKI sensitivity of diabetic or hyperglycemic cells and tissues? We first determined the effect of p53 inhibition in RPTC cells. RPTC cells were stably transfected with HA-tagged dominant negative p53 (HA-DN-p53) or a control empty vector (Supplementary Figure 4). The cells were subsequently cultured in media containing normal or high glucose, followed by azide treatment to induce ATP-depletion. In empty vector-transfected cells, azide induced 60% apoptosis following high glucose-conditioning, whereas 20% in normal glucose cells, confirming the injury sensitizing effect of high glucose (Figure 7A: Vector). Importantly, the sensitizing effect of high glucose was largely diminished in DN-p53 transfected cells (Figure 7A: HA-DN-p53). DN-p53 also suppressed the enhancing effect of high glucose on caspase activation during azide treatment (Figure 7B). Similar inhibitory effects were demonstrated for pifithrin- α , a pharmacological inhibitor of p53 (Figure 7C, 7D).

Pifithrin- α and p53 siRNA attenuate the sensitivity of Akita diabetic mice to ischemic AKI

Pifithrin- α significantly reduced ischemic AKI in Akita diabetic mice as shown by BUN and serum creatinine measurements, while its effect in wild-type, non-diabetic mice was marginal (Figure 8A, 8B). In histology, pifithrin- α did not significantly ameliorate the

severe tubular damage in diabetic tissues (Figure 8c), yet it inhibited apoptosis in diabetic tissues (Figure 8D), supporting a role of p53 in renal apoptosis in kidneys.

p53-specific siRNAs protect against AKI in animal models and are being tested in clinical trials. To further verify the role of p53 in AKI sensitivity of diabetic mice, we examined the effect of p53 siRNA on ischemic AKI in the Akita model. Akita diabetic mice and wide-type/non-diabetic littermates were injected with p53 siRNA or control GFP siRNA at 3 hours post-ischemia. Kidney tissues were collected after reperfusion to analyze p53 expression. As expected, p53 was markedly induced by ischemic AKI in Akita diabetic mice and this induction was suppressed by p53 siRNA (Supplementary Figure 5: lanes 4-6 vs. 8-10). Importantly, p53 siRNA significantly reduced BUN and serum creatinine in Akita diabetic mice, while its effect in wild-type, non-diabetic mice was less evident (Figure 9A, 9B). p53 siRNA also ameliorated the severe kidney tissue damage induced by ischemia in Akita diabetic mice (Figure 9C, 9D). In addition, it suppressed renal apoptosis in diabetic mice kidney tissues (Figure 9E, 9F).

p53 knockout from proximal tubules attenuates the sensitivity of STZ-diabetic mice to ischemic AKI

Recent research has suggested that p53 in different cell types may play distinct roles in AKI (26). To determine the cellular origin of p53 that contributes to the AKI sensitivity of diabetes, we examined the proximal tubule-specific p53 knockout mouse model (PT-p53-KO) that was established in our recent work (25). PT-p53-KO mice and their wild-type (PT-p53-WT) littermates were injected with STZ at 3 weeks of age to induce diabetes. The induction of diabetes was confirmed 3-4 weeks later by measuring blood glucose and the mice were then subjected to ischemic AKI. p53 expression during ischemic AKI was notably lower in PT-p53-KO kidney tissues than wild-type, verifying p53 ablation from proximal tubules in the knockout mice (Supplementary Figure 6). Functionally, 20 minutes of bilateral renal ischemia followed by 48 hours of reperfusion induced an significant increase of BUN and serum creatinine in wild-type diabetic mice; in stark contrast, after the same treatment, PT-p53-KO diabetic mice had BUN and serum creatinine that were significantly lower than that of wild-type (Figure 10A, 10B). Histology and TUNEL analyses further confirmed that I/R induced significantly lower tissue damage and apoptosis in PT-p53-KO diabetic mice than that in wild-type diabetic mice (Figure 10 C-F). After I/R, kidney tissues of PT-p53-KO diabetic mice also showed less cleaved/active caspase-3, lower Bax accumulation in mitochondria, and lower cytochrome c release into cytosol (Figure 10 G). These results support a critical role of proximal tubular p53 in the AKI sensitivity of diabetes.

Insulin reduces blood glucose and AKI sensitivity in STZ-diabetic mice

To determine if hyperglycemia is the triggering factor of AKI sensitivity in diabetes, we examined the effects of insulin. After STZ-induction of diabetes, one group of mice was given insulin daily for 10 days to reduce blood glucose (Figure 11A). These mice were then subjected to renal I/R injury, along with the mice without insulin injection. Insulin attenuated the AKI sensitivity of STZ-induced diabetic mice as indicated by BUN, serum creatinine, histology, and apoptosis (Figure 11 B-F; Supplementary Figure 7). The induction

of Kim1, a kidney injury marker, appeared lower in insulin treated mice (Figure 11 G). Importantly, I/R-induced p53 accumulation and phosphorylation at serine-392 was suppressed by insulin, although p53 acetylation at Lysine-379 was not changed (Figure 11 G, H). In addition, insulin suppressed cyt.c release from mitochondria into cytosol and also showed marginally inhibitory effect on Bax accumulation in mitochondria (Figure 11 I).

Discussion

Using STZ and Akita diabetic models as well as cultured kidney cells, this study provides substantial evidence for AKI sensitivity in diabetes mellitus (DM). The injury sensitivity correlates with hyperglycemia in kidney tissues and high glucose-conditioning in kidney tubular cells. Mechanistically, the injury sensitivity involves a heightened activation of the intrinsic pathway of apoptosis that is centered on mitochondria. Notably, p53 induction during AKI is markedly higher in diabetic mice and inhibition of p53 diminishes the AKI sensitivity of these animals, supporting a critical role of p53 in AKI sensitivity in diabetes mellitus (DM).

Recent epidemiologic research suggested that AKI may trigger a much more severe disease in DM patients, resulting in significant lower rates of recovery, higher rates of mortality and progress to ESRD (8, 9). In animal models, Goor et. al. documented the greater vulnerability of STZ-induced diabetic rats to ischemic AKI (16). Kelly et al. examined the long-term effects of ischemic AKI in obese-diabetic rats and proposed postischemic inflammation as a key contributing factor in the development of chronic renal failure in this condition (17). Gao et. al. further demonstrated that the AKI sensitivity of diabetic mice can be suppressed by a TNF- α neutralizing antibody, supporting a role of TNF- α -related inflammatory response (13). Our current results suggest that, at the cellular level, the injury sensitivity can be tracked down to mitochondria. Specifically, in response to hypoxia or ATP-depletion, high glucose-conditioned renal tubular cells showed significantly higher Bax translocation to mitochondria and cytochrome c release from these organelles (Figure 5A). Moreover, renal ischemia-reperfusion induced higher Bax activation and cytochrome c release at mitochondria in diabetic kidneys than non-diabetic tissues (Figure 5B). Importantly, we further show that insulin may attenuate Bax activation and cytochrome c release from mitochondria in diabetic kidneys (Figure 11H). To our knowledge, this is the first report of the sensitization of mitochondrial pathway of apoptosis by high glucose in vitro and diabetic hyperglycemia in vivo.

It remains unclear, however, why mitochondria in high glucose-conditioned cells and hyperglycemic kidney tissues are more sensitive to “Bax attack”. Nonetheless, mitochondrial dysfunction is known as an important pathogenic factor in the development of diabetic complications, including diabetic nephropathy (3). Especially, mitochondria are the main source for the production of reactive oxygen species under these disease conditions, which contributes critically to the pathogenesis by activating the signaling pathways of cell injury and death, extracellular matrix deposition, and fibrosis (3, 27). While the activation of these processes may contribute to the acute injury sensitivity of diabetic tissues and high glucose-conditioned cells, the changes at or within the mitochondria may be more proximate. In this regard, a change of mitochondrial dynamics, which leads to fragmentation

of the organelles, has been documented during high glucose incubation of renal tubular cells and in diabetic kidney tissues (28, 29). These observations are highly relevant to the current study, because fragmented mitochondria are sensitized to “Bax attack” and cytochrome c release (30). Based on these studies and our current results, we propose that high glucose or hyperglycemia in diabetes shifts mitochondrial dynamics to fission, leading to mitochondrial fragmentation, which sensitizes mitochondria to Bax attack, cytochrome c release and apoptosis during acute injury.

Technically, our cell culture model involved 2 weeks of culturing of renal proximal tubular cells in a high glucose medium. Culturing in high glucose medium, called “conditioning”, was designed to model the in vivo situation of hyperglycemia in DM. It is noteworthy that high glucose conditioning in our study is different from the shorter period of high glucose incubation that induces acute effects in kidney cells, such as oxidative stress and apoptosis (28, 31, 32). High glucose conditioning in our study may lead to adaptation of the cells to the high glucose medium and as a result, the conditioned cells did not show higher basal levels of apoptosis; nonetheless, these cells were sensitized to acute injury (Figure 4). For the in vivo diabetic mouse models, it is important to note that the AKI sensitivity occurs within 2-4 weeks of hyperglycemia, prior to the development of the functional, structural and pathological characteristics of diabetic nephropathy such as proteinuria, renal fibrosis, and glomerular pathology. Moreover, there is a good correlation between fasting blood glucose and AKI sensitivity (Figure 3). The role of hyperglycemia in AKI sensitivity in diabetes is further supported by the in vitro study of high glucose-conditioned renal tubular cells, which showed significantly higher apoptosis upon acute injury by hypoxia or ATP-depletion (Figure 4). Notably, insulin given after STZ-induction of diabetes normalized blood glucose and abrogated AKI sensitivity in mice. Insulin further suppressed p53 activation and mitochondrial injury (Figure 11). Pinpointing hyperglycemia in AKI sensitivity suggests that, although the current study used STZ-and Akita diabetic models, the finding of AKI sensitivity may also be applicable to type 2 diabetes. Nonetheless, it is noteworthy that in addition to blood glucose control, insulin may have other effects, including anti-apoptotic and proliferative actions.

At the molecular level, our results support a role of p53 in the injury sensitivity of high glucose-conditioned cells and diabetic kidneys. Up-regulation of p53 has been reported in experimental models of DN, such as STZ-treated rats and db/db diabetic mice (33-35). In line with these studies, we detected higher p53 expression and activation (indicated by phosphorylation) in high glucose-conditioned renal tubular cells in our study (Figure 6A). p53 up-regulation was also observed in some (but not all) STZ-induced and Akita diabetic mice (Figure 6 C, D; data not shown). A notable, consistent observation from both high glucose-conditioned cells and diabetic mice is the dramatic induction of p53 by acute injury in these models (Figure 6 B, C, D). The mechanism of the heightened p53 induction in diabetic cells and tissues is currently unclear, although p53 phosphorylation at specific sites (eg. serine -392) may be involved (Figure 11G, 11H). Our subsequent, inhibitory experiments using dominant negative p53, pifithrin- α , p53 siRNA and proximal tubule p53-knockout mice have provided compelling evidence for a role of p53 in the injury sensitivity of high glucose-conditioned cells and hyperglycemic kidney tissues in diabetes (Figures 7-10).

p53 is commonly known as a tumor suppressor, which induces cell cycle arrest and cell death to antagonize tumor growth. However, in cell biology p53 may be better classified as a stress response gene (36). A role of p53 in kidney injury was originally suggested by Dagher and colleagues, who demonstrated the renoprotective effect of pifithrin- α on ischemic AKI in rats (23). We and others further established a role of p53 in cisplatin-induced AKI by using both cell culture and animal models including p53-knockout mice (37-40). In recent studies, p53 was also implicated in AKI induced by folic acid, aristolochic acid, and glycerol injection (41-43). Using conditional knockout models, our latest work further established the role of proximal tubular p53 in ischemia and nephrotoxic AKI (25). Thus, inhibition of p53 may offer a therapy for AKI (23, 24, 26, 40) and p53 siRNAs were tested for AKI treatment in clinical trials (<http://clinicaltrials.gov/ct2/results?term=I5NP&Search=Search>). The results of our current study indicate that p53 inhibition may be particularly effective for AKI therapy in diabetic patients where it can diminish the AKI sensitivity of kidney tissues.

Methods

Antibodies and Reagents

Antibodies were purchased from the following sources: polyclonal anti-p53, anti-phospho-p53-ser15 from Cell Signaling Technology (Beverly, MA), polyclonal anti-Bax (N-20) from Santa Cruz Biotechnology; and monoclonal anti-cytochrome c from BD Biosciences (San Diego, CA), the secondary antibodies for immunoblot analysis from Jackson ImmunoResearch (West Grove, PA). Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD.AFC) and 7-amino-4-trifluoromethyl coumarin (AFC) were from Enzyme Systems Products (Livermore, CA). Pifithrin- α was from EMD chemicals (Philadelphia, PA). In situ cell death detection kit was obtained from Roche Applied Science (Indianapolis, IN).

Mouse Models of Diabetes

C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME). Proximal tubule-specific p53-knockout mice were produced by crossing p53(flox/flox) mice (Jackson Laboratory) with the PEPCK-Cre mice as described in our recent work (25). The mice were backcrossed to maintain a mainly C57BL/6 background. For STZ-induction of diabetes, mice at 4-weeks of age were injected with 50 mg/kg body-weight STZ (Sigma-Aldrich, St. Louis, MO) for 5 consecutive days according to a standard protocol (44). STZ-induced mice were maintained for another 2-3 weeks before renal ischemia-reperfusion. To test the effect of insulin, 30 unit/kg LANTUS (insulin glargine, a long-acting insulin) was injected daily one week after STZ-induction for 10 days before renal ischemia-reperfusion. For Akita diabetic model, C57BL/6J-Ins2^{Akita/+} mice were obtained from Jackson Laboratory for breeding. Genotyping of offspring was performed according to the protocol recommended by Jackson Laboratory. Ins2^{Akita/+} heterozygous male mice and wild-type Ins2^{+/+} littermates (8 to 10 weeks old) were used for experiments. All animals were housed in the animal facility of Charlie Norwood VA Medical Center at Augusta with a 12/12-hour light/dark cycle and food and water available ad libitum. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Charlie Norwood VA Medical Center at Augusta. Fasting blood glucose was measured

twice a week using a glucometer after the mice were fasted for ~8 hours. The values presented in this study (Figure 3) were from the measurement prior to renal ischemia study. The animals with > 200 mg/dL fasting blood glucose for two consecutive readings were considered diabetic.

Renal Ischemia-Reperfusion

Renal ischemia-reperfusion was induced in mice as detailed recently (45, 46). Briefly, mice were anesthetized with 60 mg/kg (intraperitoneally) pentobarbital sodium and kept on a homeothermic station to maintain body temperature at 36.5°C. Kidneys were exposed by bilateral flank incisions, and the renal pedicles were clamped to induce ischemia. After ischemia, the clamps were released for reperfusion. Sham control animals were subjected to identical operation without renal pedicle clamping.

Cell Treatment

Immortalized rat kidney proximal tubular cell line (RPTC) was originally obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH) and maintained for experiments as described previously (30, 47). For high glucose conditioning, RPTC cells were cultured and passaged for two weeks in a medium containing 30 mM glucose, a concentration (equivalent to ~545 mg/dl) used for in vitro hyperglycemic treatment (28). The control groups of cells were grown in media containing 5.5 mM glucose (100 mg/dl, normal glucose) or 5.5 mM glucose with 24.5 mM mannitol. For azide treatment, RPTC cells were incubated for 3 hours in a glucose-free basic buffer with 10 mM azide that blocks cellular respiration at complex IV to induce ATP-depletion. The cells were then returned to full culture medium for 2 hours to develop apoptotic morphology (47, 48). For hypoxic treatment, RPTC cells were incubated in the glucose-free Krebs-Ringer bicarbonate buffer for 4 hours in a hypoxic chamber with undetectable oxygen. After hypoxic incubation, the cells were returned to full culture medium for “reperfusion” to develop apoptotic morphology (49).

Renal Function, Histology and Apoptosis

BUN and serum creatinine were determined using commercial kits to indicate renal function as previously (45-47). Renal histology was examined by hematoxylin and eosin staining. Histo-pathological changes were evaluated by the percentage of injured/damaged renal tubules as indicated by tubular lysis, dilation, disruption, and cast formation. Tissue damage was scored as follows: 0, no damage; 1, <25%; 2, 25 to 50%; 3, 50 to 75%; and 4, >75%. Renal apoptosis was examined by TUNEL assay using the in situ Cell Death Detection kit from Roche Applied Science (Indianapolis, IN). Briefly, paraffin-embedded renal tissue sections were deparaffinized and permeabilized by 30 minutes of incubation at 37°C in 0.1 M sodium citrate (pH 6.0). The sections were then exposed to the TUNEL reaction mixture containing TM red-labeled dUTP. TUNEL positive nuclei were identified by fluorescence microscopy.

Apoptosis and Caspase Analysis

Apoptosis in RPTC cells was examined morphologically as described in our previous work (30, 47). Briefly, cells were stained with Hoechst 33342 and examined by phase-contrast and fluorescence microscopy. Apoptotic cells were identified by characteristic morphology including cellular condensation, formation of apoptotic bodies, and condensation and fragmentation of the nucleus. About 200 cells were examined in each dish to determine the percentage of apoptotic cells. Caspase activity in cell lysate was measured by the enzymatic assay using DEVD. AFC as the reaction substrate (30, 47).

Isolation of cytosolic and mitochondrial fractions

RPTC cells were fractionated by using digitonin to investigate the subcellular redistributions of Bax and cytochrome c (30, 50). Briefly, cells were permeabilized with 0.05% (wt/vol) digitonin in an isotonic sucrose buffer for 2–4 minutes at room temperature. The released fraction was collected as cytosol and the digitonin-insoluble part was further extracted with 2% SDS to collect the membrane-bound organellar fraction enriched with mitochondria. For kidney tissues, mitochondrial and cytosolic fractions were collected as described in our recent work (20). Briefly, fresh or frozen kidney tissues were homogenized in a buffer containing 0.27 M sucrose, 1mM EGTA, and 5 mM Tris-HCl (pH 7.4). The homogenates were first centrifuged at 600 g for 10 minutes to remove cell debris and nuclei, and then centrifuged at 10,000 g for 10 minutes to collect the pellet as mitochondrial fraction. The supernatant was further centrifuged at 100,000 g for 60 minutes to collect the cytosolic fraction. The whole isolation procedure was conducted at 4°C.

Immunoblot Analysis

Protein concentration was determined using the BCA reagent (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein were loaded in each well for electrophoresis using the NuPAGE Gel System (Invitrogen, Carlsbad, CA), followed by transferring onto polyvinylidene fluoridemembranes. The blots were then incubated sequentially in blocking buffer for 1 hour, primary antibodies overnight at 4°C, and the horseradish peroxidase-conjugated secondary antibodies. The antigens on the blots were revealed by using the enhanced chemiluminescence (ECL) kit from Thermo Fisher Scientific (Waltham, MA).

Statistical Analysis

Kaplan-Meier analysis was conducted using the GraphPad Prism software. Statistical differences between two groups were determined by two-tailed unpaired t-test (Microsoft Excel). $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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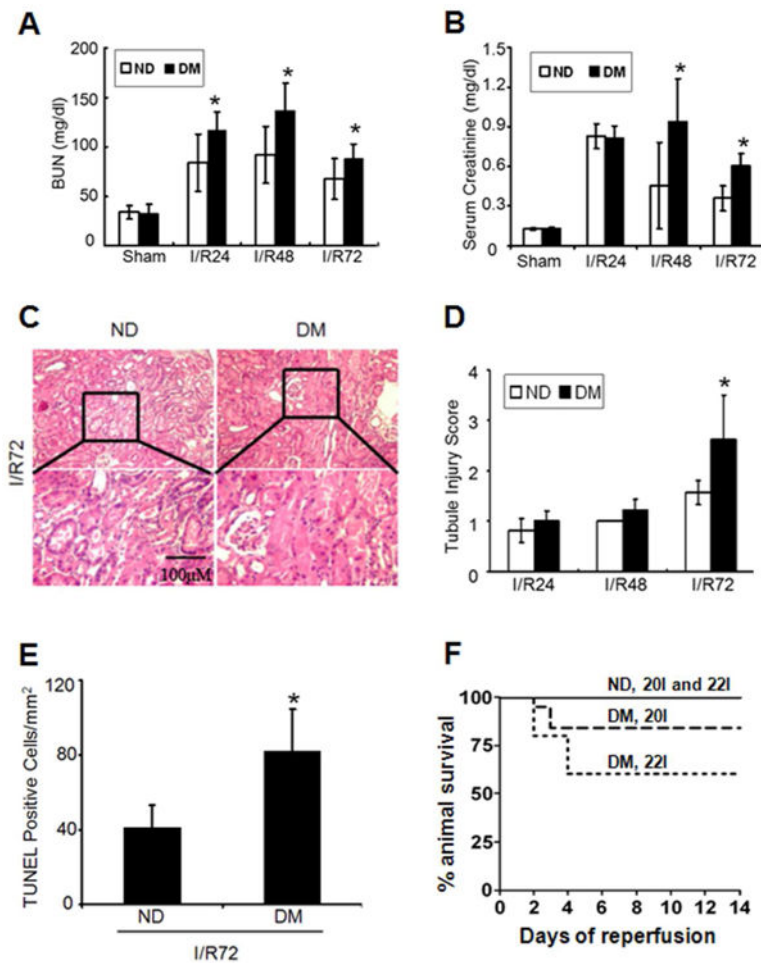


Figure 1. Sensitivity of STZ-induced diabetic mice to ischemic AKI

(A-E) Male C57BL/6J mice were injected with STZ to induce diabetes (DM) or injected with vehicle solution (ND: non-diabetic). The mice were then subjected to sham operation or 20 minutes of bilateral renal ischemia followed by 24, 48, or 72 hours of reperfusion (I/R24, I/R48, I/R72). Blood samples were collected at indicated time points to measure BUN (A) and serum creatinine (B). Renal tissues were collected for hematoxylin and eosin staining to record representative histology (C) and semi-quantify tissue damage (D). Renal cortical and outer medulla tissues were also examined by TUNEL assay to reveal apoptosis (E). The data in (A, B, D, E) are means \pm SD (n=4-8); *, $p < 0.05$ vs. the relevant ND group. (F) Survival of STZ-induced diabetic mice and non-diabetic mice after ischemic AKI. STZ-induced diabetic mice and non-diabetic mice were subjected to 20 or 22 minutes of bilateral renal ischemia (20I, 22I) followed by reperfusion. Animal survival was recorded for 2 weeks.

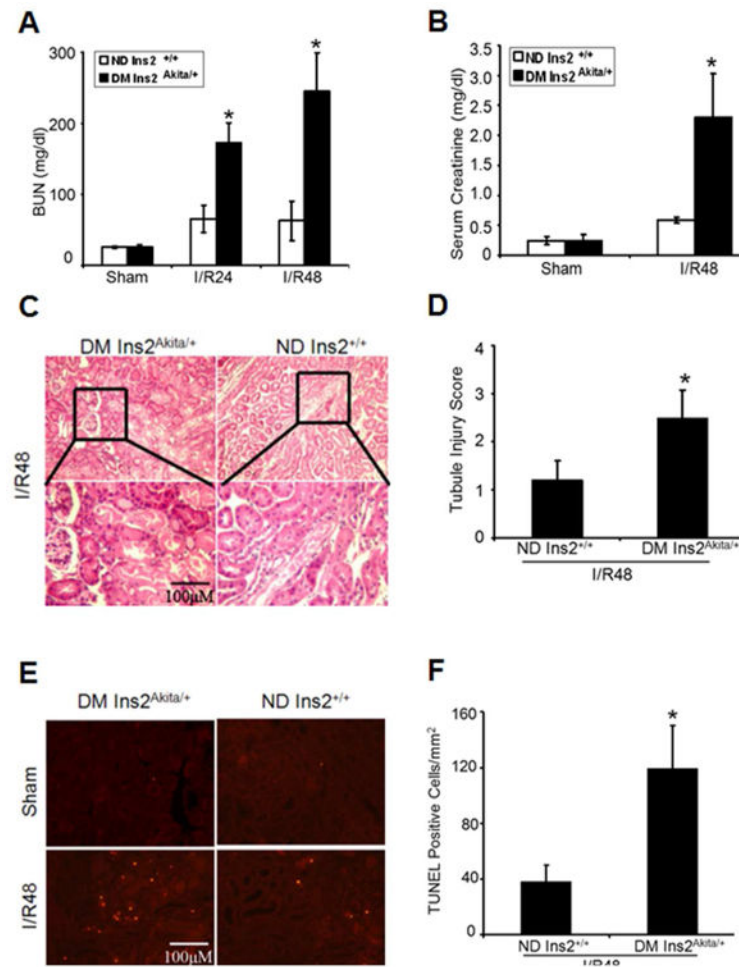


Figure 2. Enhanced ischemic AKI in Akita diabetic mice

Male Akita diabetic (DM Ins2^{Akita/+}) and wide type non-diabetic (ND Ins2^{+/+}) mice of 8-10 weeks of age were subjected to sham operation or 23 minutes of bilateral renal ischemia followed by 24 or 48 hours of reperfusion (I/R24, I/R48). Blood samples were collected at indicated time points to measure BUN (A) and serum creatinine (B). Renal cortical and outer medulla tissues were collected for hematoxylin and eosin staining to record representative histology (C) and semi-quantify tubular damage (D). The tissues were also examined by TUNEL assay to record and quantify apoptosis (E, F). The data in (A, B, D, F) are means \pm SD (n=4-8); *, p<0.05 vs. the relevant ND-Ins2^{+/+} group.

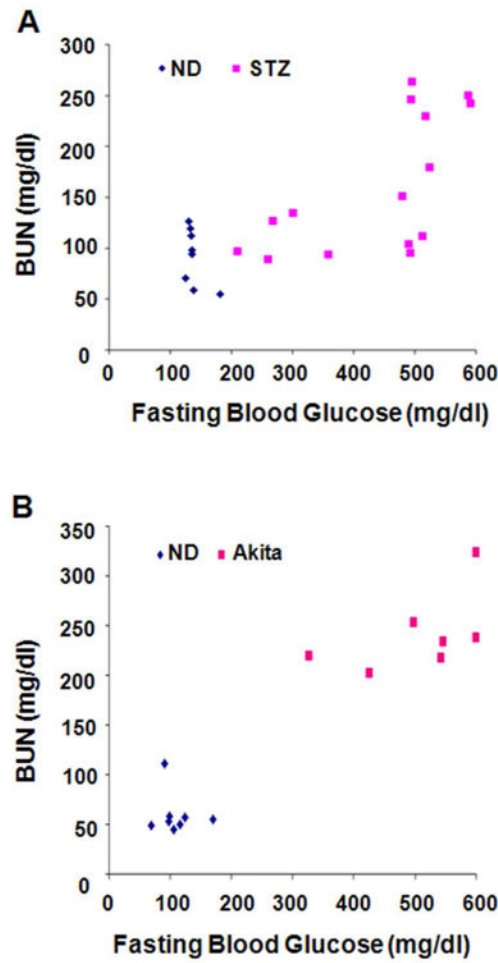


Figure 3. Correlation between ischemic AKI and fasting glucose in diabetic and non-diabetic mice

Fasting blood glucose was recorded for the animals used in the experiments described in Figures 1 (A) and 2 (B) and plotted against the BUN values of these animals after renal ischemia-reperfusion (48 hours). The SPSS 13.0 software was used to analyze the correlation between fasting blood glucose and BUN.

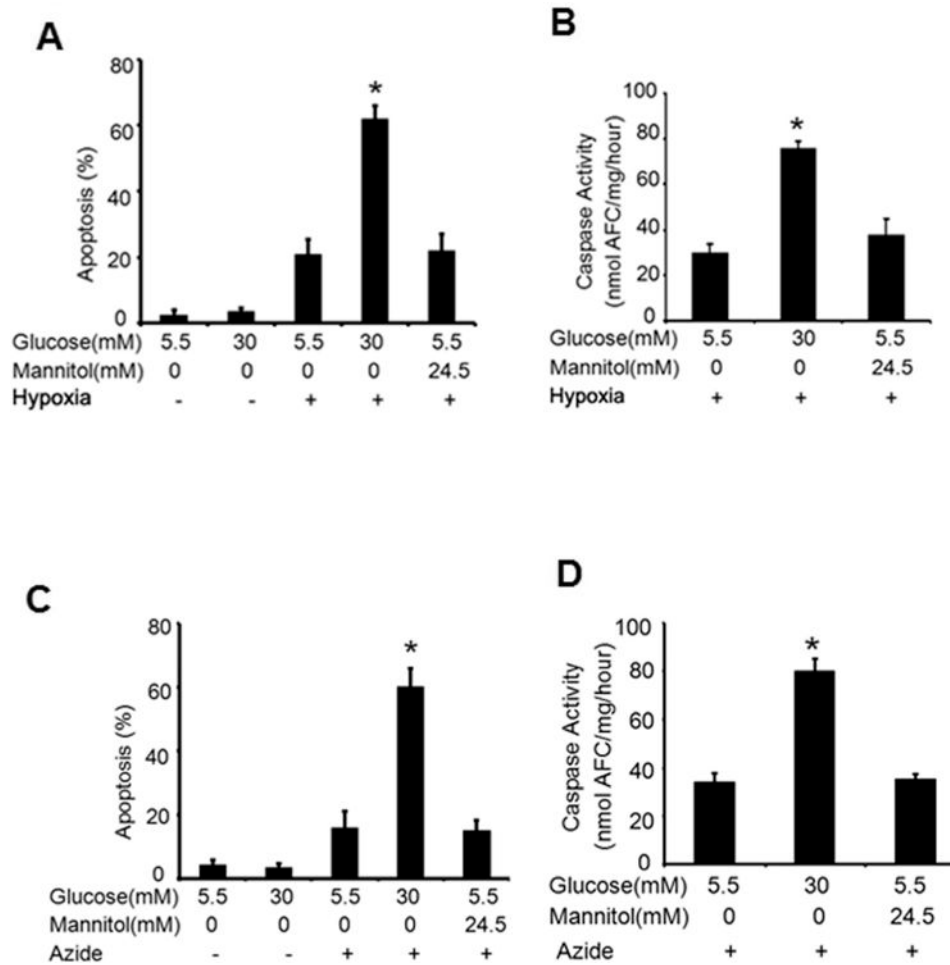


Figure 4. High glucose-conditioned RPTC cells are sensitized to apoptosis following ATP-depletion and hypoxia

RPTC cells were cultured for two weeks in media containing 5.5 mM glucose, 30 mM glucose, or 5.5 mM glucose+24.5 mM mannitol. The cells were then subjected to 4 hours of severe hypoxia (**A, B**) or 3 hours of azide treatment to induce ATP-depletion (**C, D**), followed by 2 hours reperfusion. Apoptosis was evaluated by nuclear and cellular morphology after Hoechst staining (**A, C**). Cell lysate was collected to measure caspase activity by an enzymatic assay (**B, D**). Data are presented as mean \pm SD; n = 3. *P < 0.05 vs. 5.5 mM glucose groups.

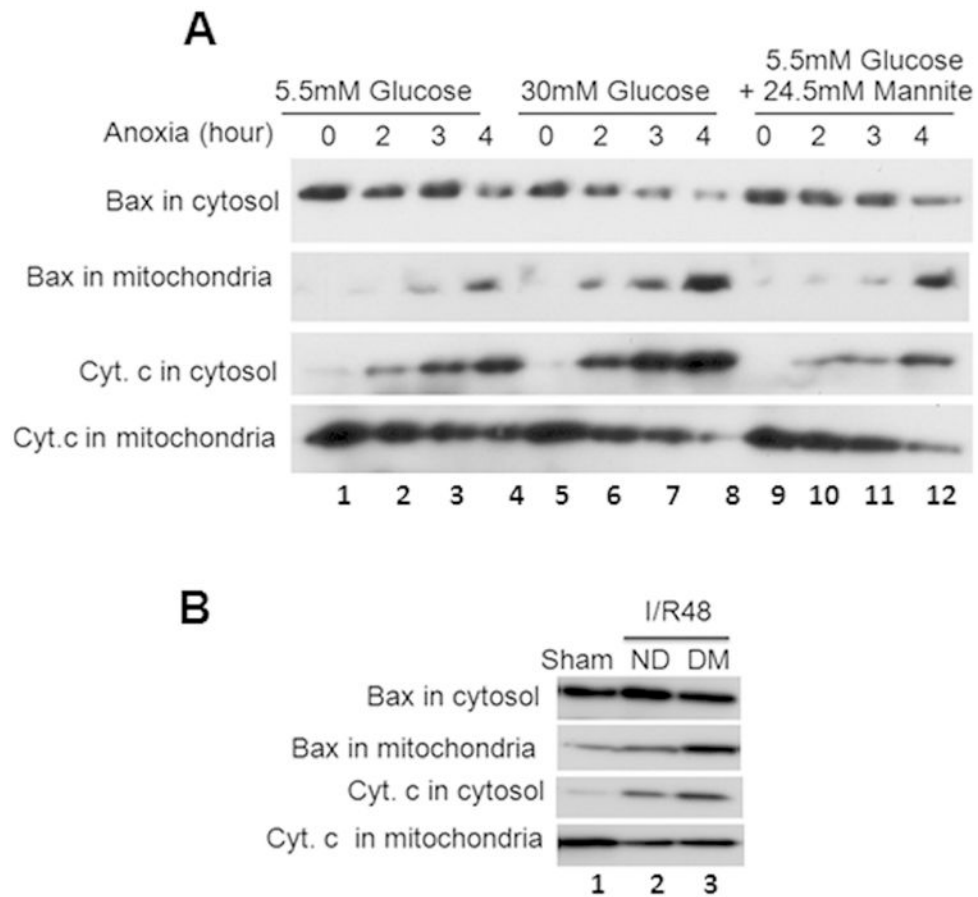


Figure 5. Bax translocation and cytochrome c release in highglucose-conditioned RPTC cells and Akita diabetic mice

(A) RPTC cells were cultured for two weeks in media containing 5.5 mM glucose, 30 mM glucose, or 5.5 mM glucose+24.5 mM mannitol. The cells were then subjected to 4 hours of severe hypoxia and fractionated into cytosolic and membrane-bound fraction with mitochondria for immunoblot analysis of Bax and cytochrome c. (B) Akita diabetic (DM-Ins2^{Akita/+}) and wild-type non-diabetic (ND-Ins2^{+/+}) mice were subjected to sham operation or 23 minutes bilateral renal ischemia followed by 48 hours of reperfusion. Renal tissues were homogenized and fractionated into cytosolic and mitochondrial fractions for immunoblot analysis of Bax and cytochrome c.

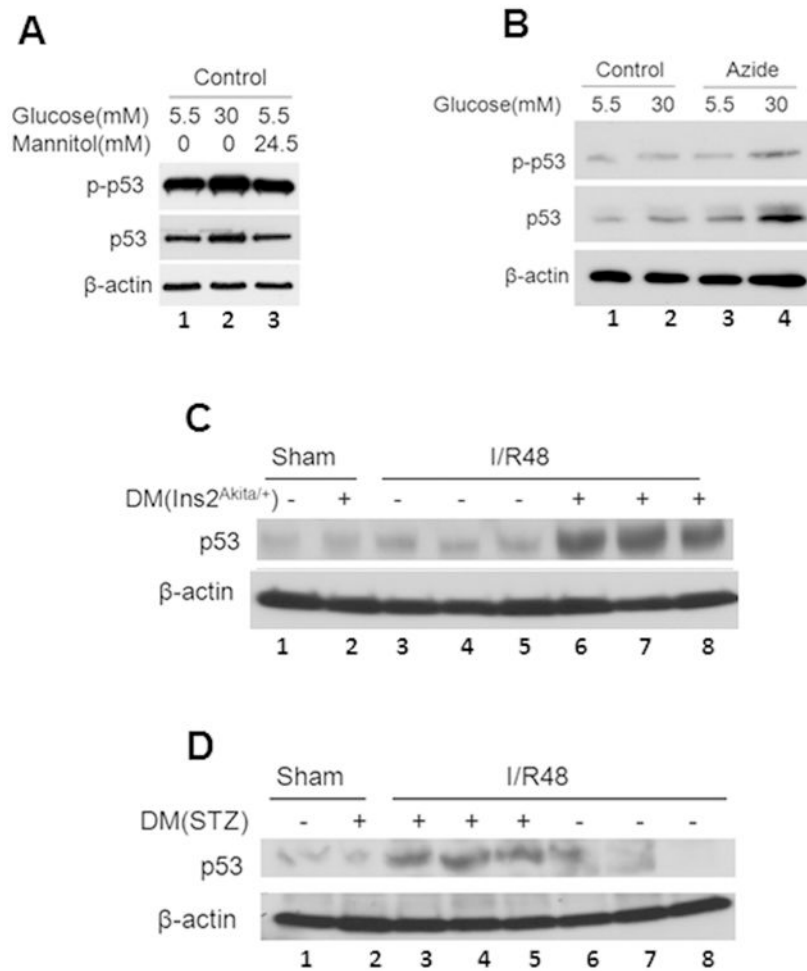


Figure 6. Heightened p53 induction in high glucose-conditioned RPTC cells and diabetic kidneys (A) Whole cell lysate was collected from RPTC cells that were cultured with 5.5 mM glucose, 30 mM glucose, or 5.5 mM glucose+24.5 mM mannitol for immunoblot analysis of serine-15 phosphorylated p53 (p-p53), total p53, and β -actin. (B) RPTC cells cultured with 5.5 mM glucose or 30 mM glucose were left untreated (Control) or subjected to azide-induced ATP-depletion followed by recovery. Whole cell lysate was collected for immunoblot analysis of p-p53, total p53, and β -actin. (C) Akita diabetic mice (-) and wide-type non-diabetic mice (+) were subjected to sham operation or 23 minutes of renal ischemia followed by 48 hours of reperfusion. Whole tissue lysate was collected for immunoblot analysis of p53 and β -actin. (D) C57BL/6 mice were treated with (+) or without (-) STZ and then subjected to sham operation or 20 minutes of renal ischemia followed by 48 hours of reperfusion to collect whole tissue lysate for immunoblot analysis of p53 and β -actin.

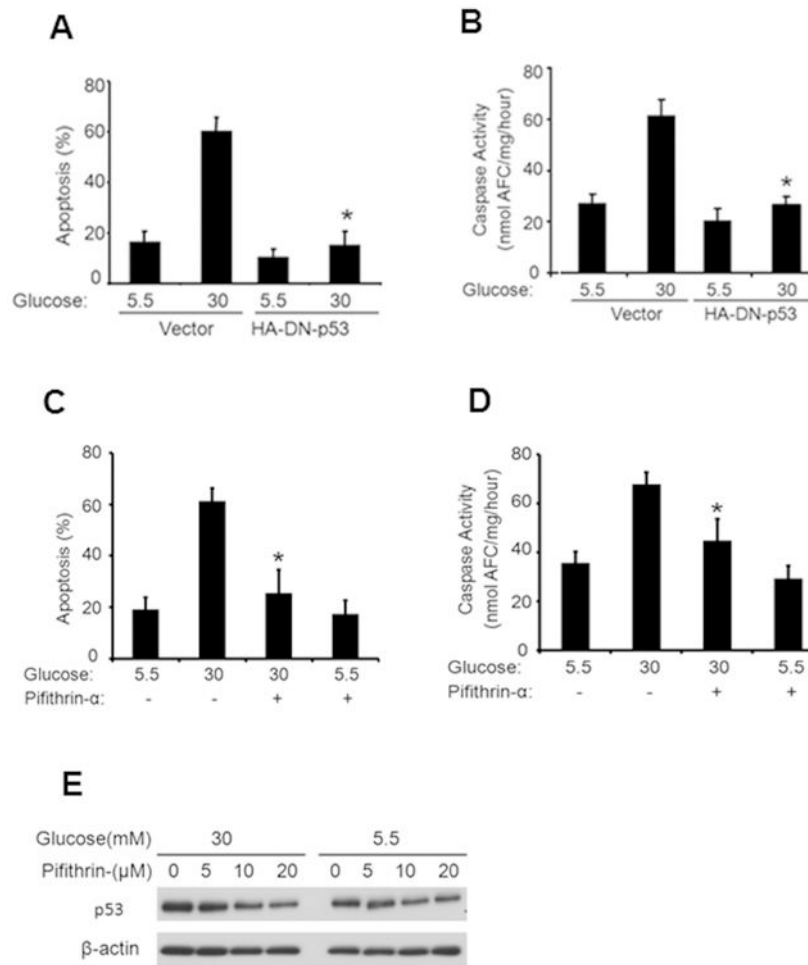


Figure 7. Inhibition of p53 reduces ATP-depletion-induced apoptosis in high glucose-conditioned cells

(**A, B**) RPTC cells stably transfected with HA-tag dominant negative p53 (HA-DN-p53) or empty vector were cultured in media containing 5.5 or 30 mM glucose. The cells were then subjected to azide-induced ATP-depletion followed by 2 hours of recovery in full culture medium to analyze apoptosis (**A**) and caspase activity (**B**). (**C, D**) RPTC cells were cultured in media containing 5.5 or 30 mM glucose. The cells were then subjected to azide-induced ATP-depletion followed by 2 hours of recovery with (+) or without (-) 20 μ M pifithrin- α to analyze apoptosis (**C**) and caspase activity (**D**). Data are means \pm SD. n = 4. * p<0.05 vs. Vector/30mM glucose group in (**A, B**) or DMSO/30mM glucose group (**C, D**).

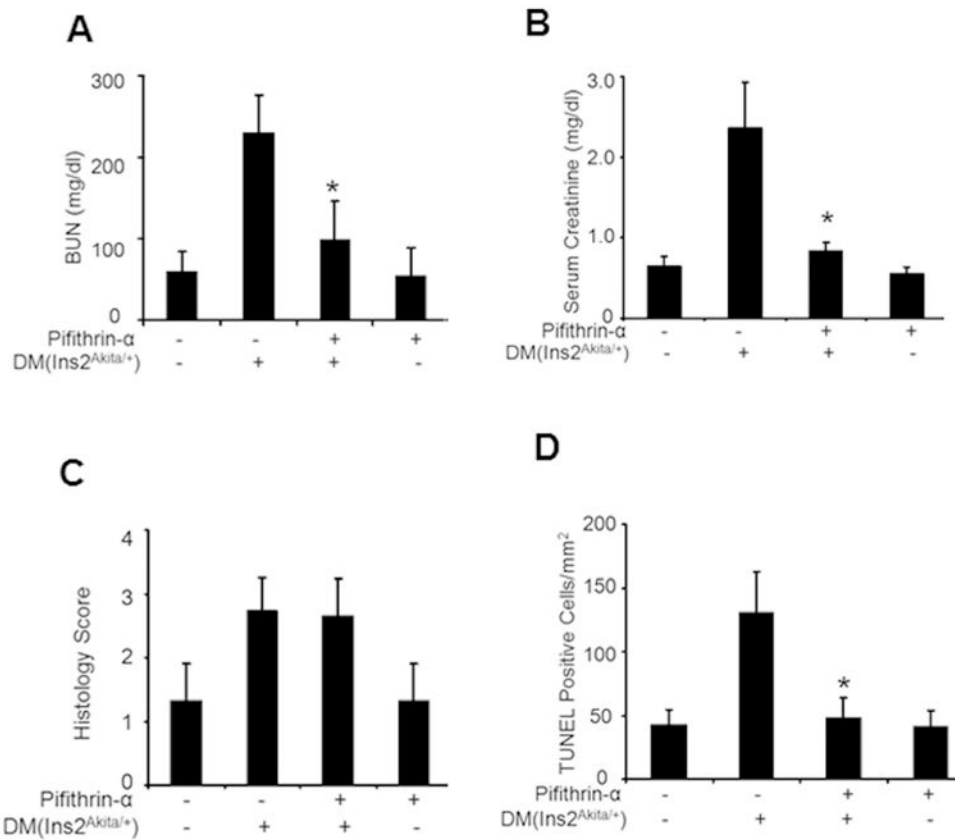


Figure 8. Pifithrin- α attenuates the AKI sensitivity in Akita diabetic mice

Akita diabetic (DM-Ins2^{Akita/+}) mice and wide-type non-diabetic (ND-Ins2^{+/+}) mice were injected with 2 mg/kg body weight pifithrin- α or saline, and then subjected to 23 minutes of bilateral renal ischemia followed by 48 hours of reperfusion. Blood samples were collected to measure BUN (A) and serum creatinine (B). Renal cortical and outer medulla tissues were collected for hematoxylin and eosin staining to examine histology (C) and for TUNEL assay of apoptosis (D). Data are means \pm SD. n = 3. * p < 0.05 vs. DM-Ins2^{Akita/+}/saline group.

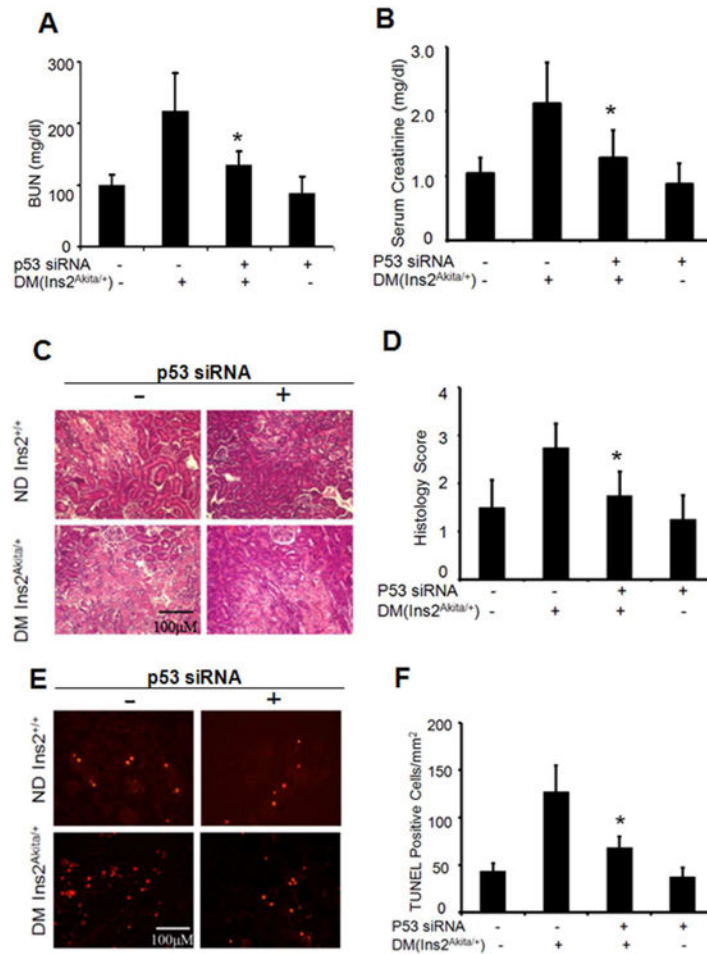


Figure 9. p53 siRNA attenuates the AKI sensitivity in Akita diabetic mice
 Akita diabetic (DM-Ins2^{Akita/+}) mice and wide-type non-diabetic (ND-Ins2^{+/+}) mice were subjected to 23 minutes of bilateral renal ischemia followed by 48 hours of reperfusion. p53 siRNA or GFP siRNA were injected at 15 mg/kg body weight 3 hours post-ischemia. Blood samples were collected to measure BUN (A) and serum creatinine (B). Renal cortical and outer medulla tissues were collected for hematoxylin and eosin staining to record representative histology and grade tubular injury (C, D) for for TUNEL assay of apoptosis (E, F). Quantitative data are means ± SD. n = 3. * p<0.05 vs. DM-Ins2^{Akita/+}/saline group.

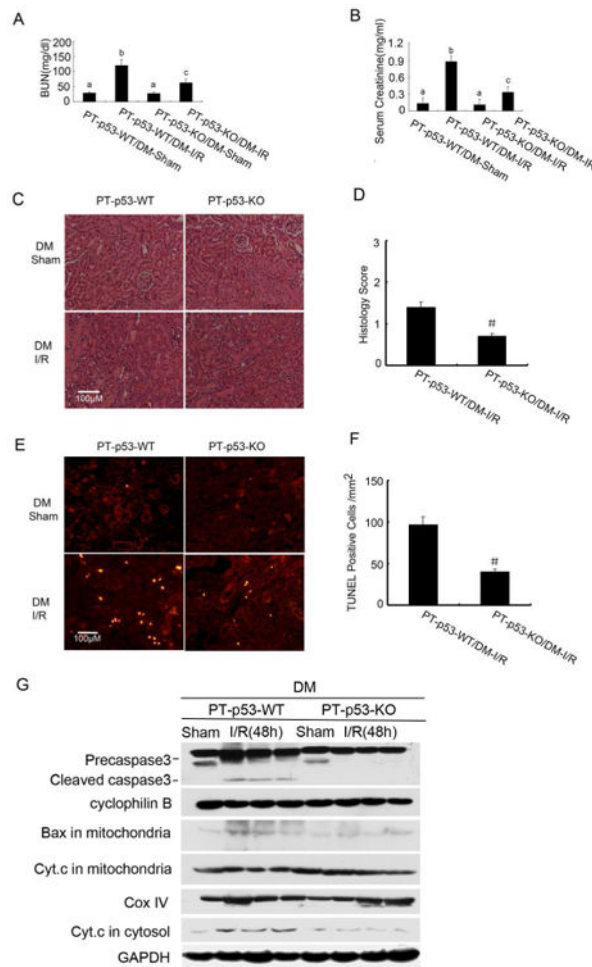


Figure 10. p53 ablation from proximal tubules attenuates AKI sensitivity in STZ-diabetic mice
 Male proximal tubule-specific p53 knockout (PT-p53-KO) mice and wild-type (PT-p53-WT) littermates of 3 weeks of age were injected with STZ to induce diabetes and then subjected to 20 minutes of bilateral renal ischemia followed by 48 hours of reperfusion (I/R) or sham operation as control. (A, B) Blood samples were collected to measure BUN and serum creatinine. (C, D) Kidney cortical and outer medullar tissues were stained with hematoxylin-eosin to record histology and grade tubular damage. (E, F) Kidney tissues were examined by TUNEL assay to record representative images of and quantify apoptosis. (G) Kidney cortical tissues were homogenized for immunoblot analysis of caspase 3 or fractionated to isolate mitochondrial and cytosolic fractions for immunoblot analysis of Bax and Cyt c. Cyclophilin B and Cox IV were probed as for loading controls for whole lysate or mitochondrial fraction, respectively. Quantitative data are expressed as mean±SD, n=8; * p<0.05 vs. PT-p53-WT/DM-I/R group.

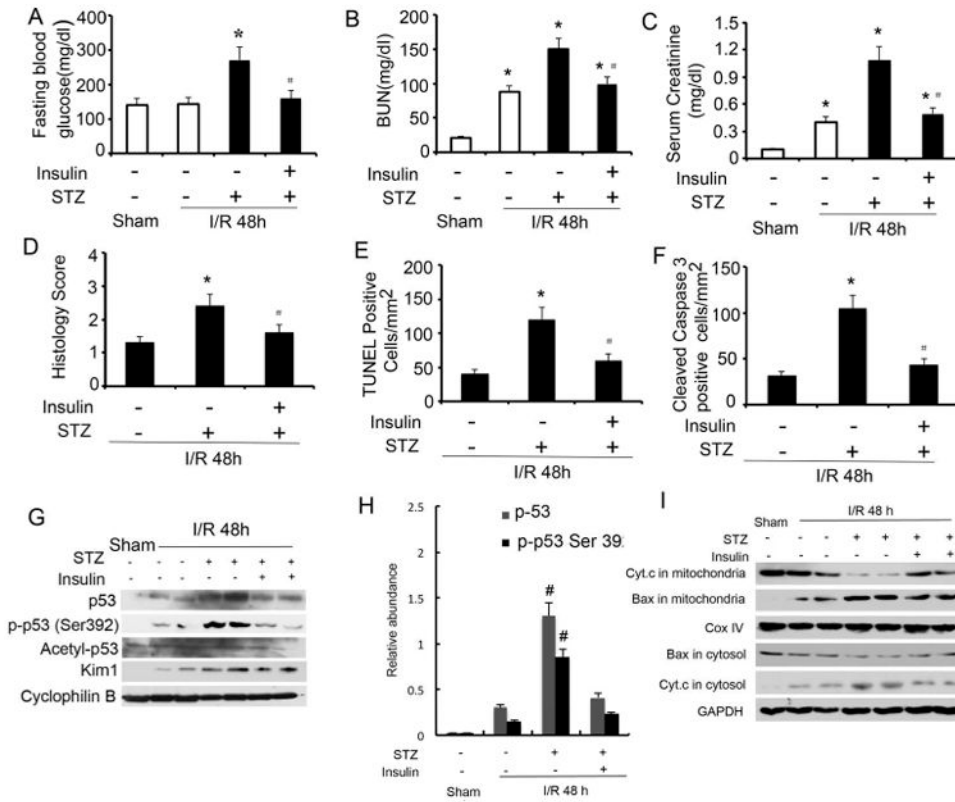


Figure 11. Insulin reduces blood glucose and AKI sensitivity in STZ-induced diabetic mice
 Male C57BL/6J mice were injected with STZ to induce diabetes or injected with vehicle solution. One week later, some STZ-induced mice were given 30 unit/kg of insulin daily for 10 days, while others were injected with vehicle solution. Fasting blood glucose was measured ~24 hours after insulin injection to indicate the effect of insulin on hyperglycemia in STZ-induced mice (A). The mice were then subjected to sham operation or 23 minutes of bilateral renal ischemia followed by 48 hours of reperfusion (I/R48h). Blood samples were collected to measure BUN and serum creatinine (B, C). Renal tissues were collected for hematoxylin and eosin staining to examine histological damage score (D), for TUNEL assay of apoptosis (E), and for immunohistochemical staining of active/cleaved caspase 3 (F). (G) Whole tissue lysate was analyzed for p53, p-p53(ser-392), Acetyl-p53(Lys-379), Kim1, and cyclophilin B (loading control). (H) Densitometric analysis of p53 and p-p53(ser-392) signals relative to cyclophilin B. (I) Kidney tissues were fractionated into mitochondrial and cytosolic fractions for immunoblot analysis of cyt. c, Bax, Cox IV (mitochondrial loading control), and GAPDH (cytosolic loading control). Quantitative data in this figure are means ± SD (n=4-8); * p<0.05 vs. Sham; #, p<0.05 vs. STZ +I/R48 without insulin group.