Activation of glycogen synthase kinase 3β ameliorates diabetes-induced kidney injury* 

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Background: High glucose-induced matrix protein synthesis in renal cells requires glycogen synthase kinase 3β (GSK3β) inactivation. 

Results: Sodium nitroprusside (SNP) activated GSK3β and inhibited diabetes-induced kidney hypertrophy, matrix deposition, and albuminuria in mice without changing blood glucose. 

Conclusion: Activation of GSK3β by SNP ameliorates diabetic kidney injury. 

Significance: GSK3β may be a novel target for intervention in diabetic kidney disease. 

ABSTRACT 

Increase in protein synthesis contributes to kidney hypertrophy and matrix protein accumulation in diabetes. We have previously shown that high glucose (HG)-induced matrix protein synthesis is associated with inactivation of glycogen synthase kinase 3β (GSK3β) in renal cells and in the kidneys of diabetic mice. We tested if activation of GSK3β by sodium nitroprusside (SNP) mitigates kidney injury in diabetes. Studies in kidney proximal tubular epithelial cells showed that SNP abrogated HG-induced laminin increment by stimulating GSK3β and inhibiting Akt, mTORC1, and events in mRNA translation regulated by mTORC1 and Erk. NONOate, an NO donor, also activated GSK3β indicating that NO may mediate SNP stimulation of GSK3β. SNP administered for 3 weeks to mice with streptozotocin-induced type 1 diabetes ameliorated kidney hypertrophy, accumulation of matrix proteins and albuminuria without changing blood glucose levels. Signaling studies showed...
diabetes caused inactivation of GSK3β, by activation of Src, Pyk2, Akt, Erk; GSK3β inhibition activated mTORC1 and downstream events in mRNA translation in the kidney cortex. These reactions were abrogated by SNP. We conclude that activation of GSK3β by SNP ameliorates kidney injury induced by diabetes.

Introduction

Diabetes is the major cause of end stage renal disease (ESRD) (1,2). Diabetic kidney disease is characterized by excessive deposition of extracellular matrix (ECM) in the form of thickening of glomerular and tubular basement membranes and increased amount of mesangial matrix (glomerulosclerosis) and tubulo-interstitial fibrosis (3). We have previously reported that high glucose and high insulin, conditions associated with type 2 diabetes, increased protein synthesis including matrix proteins in the renal proximal tubular epithelial (MCT) cells (4). These changes were associated with inactivation of glycogen synthase kinase 3beta (GSK3β), a ubiquitously expressed and constitutively active serine/threonine kinase (5). GSK3β regulates a variety of cellular processes including glycogen metabolism (6), gene transcription (7), apoptosis (8) and microtubule stability (9,10); our studies have shown that it serves as a constitutive inhibitor of protein synthesis in renal epithelial cells (5). Akt promotes protein synthesis by phosphorylating GSK3β at Ser-9 thereby inhibiting its activity (11,12). GSK3β activity can also be regulated by Tyr-216 phosphorylation (13,14). GSK3β regulates the activity of a broad range of substrates by phosphorylation, e.g., glucose metabolism by phosphorylation and inactivation of glycogen synthase (15) and protein synthesis by phosphorylation and inhibition of eukaryotic initiation factor 2B epsilon (eIF2Bε) (5,16). eIF2B is a heteropentamer; its catalytic ε subunit promotes GDP/GTP exchange on eIF2, a key regulatory step in the initiation phase of mRNA translation (17). Augmented protein synthesis contributes to kidney hypertrophy and matrix protein increment seen in diabetic kidney disease (18). Although GSK3β is an inhibitor of high glucose-induced protein synthesis, whether its activation ameliorates diabetic kidney disease in vivo has not been studied. Sodium nitroprusside (SNP) is a GSK3β activator (19). In this study, we investigated if activation of GSK3β by SNP ameliorates diabetes-induced kidney hypertrophy, albuminuria and matrix protein accumulation.

EXPERIMENTAL PROCEDURES

Cell culture. SV-40 immortalized murine kidney proximal tubular epithelial (MCT) cells (kindly provided by Dr. Eric Neilson, Northwestern University) were grown in DMEM containing 7% fetal bovine serum, 5 mM glucose, 100 u/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Confluent cells were growth-arrested for 18h in serum-free DMEM before experiment (20).

Animal study. Our Institutional Animal Care and Use Committee approved the studies. Diabetes was induced in mice as described (http://www.diacomp.org/shared/protocols.aspx?model=4). C57Bl6 mice received daily intra-peritoneal injections of streptozotocin (STZ; 50 mg/kg) for 5 days (20); control mice received the citrate buffer. Following the determination of optimal dose of sodium nitroprusside (SNP), control and diabetic mice received SNP 200 µg/kg or its vehicle intraperitoneally daily for 3 weeks.

Albuminuria estimation. Mice were individually placed in metabolic cages prior to sacrifice and urine was collected for 24 hr. Commercial kits were used to measure albumin (Bethyl Laboratories, Montgomery, TX) and creatinine (Enzo Life Sciences Inc, Farmingdale, NY) (21).
Immunoblotting. Primary antibodies were from Cell Signaling (Beverly, MA) if not otherwise mentioned. Laminin-β1 and GSK3β antibodies were from SantaCruz Biotechnology (SantaCruz, CA), fibronectin antibody was from Sigma (St.Louis, MO) and phospho-eIF2Bε (Ser539) was from Upstate (Lake Placid, NY). Immunoblotting and scanning of the bands were done as previously described (20,22).

Immunohistochemical analysis. Cryosections of snap-frozen kidney tissues from control and diabetic mice treated with or without SNP, were subjected to immunoperoxidase (IP) staining with a polyclonal antibody against laminin trimer from Thermo-NeoMarker (Waltham, MA) as described (23). The area of laminin staining within the glomerulus was measured in digital images by selecting a lower and upper range of gray scale within the limits of background and the highest intensity of laminin IP staining. The circumference of each glomerulus was outlined and the specific staining area was identified by pseudocoloring and calculated as a percentage of total glomerular area as described earlier (24). The measurements were made using a computer-based morphometric analysis system with Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD).

Statistical analysis. All values are expressed as mean ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA) for comparison between multiple groups and post-hoc analysis using Newman Keul’s multiple comparison tests using Graph Pad Prism 4 software. Statistical comparisons between two groups were performed by the Student’s t-test. Statistical significance was assigned to values of p<0.05.

RESULTS

High glucose (HG) inactivates GSK3β. HG significantly increased Ser9 phosphorylation and reduced Tyr216 phosphorylation of GSK3β (Fig. 1A); this was associated with Ser535 dephosphorylation of eIF2Bε (Fig. 1A); both these changes indicated GSK3β inactivation by HG. The changes in phosphorylation were seen within 10 to 15 min and sustained for nearly 60 min. HG increased GSKα Ser21 phosphorylation but did not affect its Tyr279 phosphorylation (Fig. 1A).

SNP inhibits HG-induced changes in phosphorylation of GSK3β and eIF2Bε. SNP has been employed as a GSK3β activator in Alzheimer’s disease (25), human medulloblastoma (26) and cholangiocarcinoma (19). Dose response studies showed that 50 µM of SNP was sufficient to activate GSK3β as indicated by reduced Ser9 phosphorylation and increased Ser535 phosphorylation of its substrate eIF2Bε (Fig. 1B). Pre-incubation of MCT cells with SNP for 30 min. abrogated HG-induced changes in phosphorylation of GSK3β and eIF2Bε (Fig. 1C).

SNP inhibits HG-induced matrix protein expression and upstream regulators of GSK3β. HG rapidly increased the expression of matrix proteins laminin β1 and laminin γ1 that was blocked by SNP (Fig. 2A, B). Activated Akt in response to high glucose inhibits GSK3β by phosphorylating it at Ser9 (5,27). In addition, we have identified Erk 1/2 MAP kinase as an upstream regulator of GSK3β Ser9 phosphorylation in MCT cells (6). SNP pre-treatment blocked HG-induced phosphorylation and activation of Akt and Erk (Fig. 2C, D), suggesting that SNP activation of GSK3β is mediated by inhibition of Akt and Erk in HG treated cells.

SNP blocks HG-induced activation of Src and Pyk2, kinases that regulate Erk and Akt activity. To identify the link between hyperglycemia and GSK 3β we explored Src and Pyk2, which have been reported as upstream regulators for Akt and Erk (22,28,29). HG increased Tyr416 autophosphorylation of Src and Tyr402 autophosphorylation of Pyk2.
phosphorylation of Pyk2, a calcium-dependent proline-rich non-receptor tyrosine kinase (Fig. 3A, B). SNP inhibited HG-induced phosphorylation of Src and Pyk2 (Fig. 3A, B). Next we examined if activation of Src and Pyk2 is required for HG-induced GSK3β inactivation and laminin γ1 synthesis. Pre-incubation with either PP2, a Src inhibitor, or, BAPTA/AM, a calcium chelator that inhibits calcium-dependent Pyk2, blocked HG-induced phosphorylation of GSK3β (Fig. 3C, D, respectively) and laminin γ1 synthesis (Fig. 3E, F, respectively). Furthermore, activation of Src and Pyk2 was required for HG-induced activation of Akt and Erk (Figs. 3G to 3J). These data show that HG activates Src and Pyk2 as upstream regulators of Akt and Erk leading to inhibition of GSK3β culminating in laminin synthesis; SNP inhibits these HG-induced events.

SNP abrogates stimulatory effects of HG on the initiation and elongation phases of mRNA translation. As mRNA translation is rate limiting for peptide generation, we examined if SNP affected HG stimulation of translation events. mTOR complex1 (mTORC1) regulates the initiation and elongation phases of mRNA translation by inactivation of 4EBP1 and activation of p70S6 kinase. Eukaryotic initiation factor 4E (eIF4E) is held in an inactive complex by its binding protein 4EBP1; phosphorylation of the latter releases eIF4E which undergoes phosphorylation on Ser209, and associates with eIF4G to facilitate the initiation phase of translation. p70S6 kinase phosphorylates Ser366 of eukaryotic elongation factor 2 (eEF2) kinase, resulting in its inactivation; reduced activity of eEF2 kinase contributes to reduction in Thr-56 phosphorylation of eEF2 which facilitates the elongation phase of translation (12,24). HG-induced activation of mTORC1 indicated by increased Thr389 phosphorylation of p70S6 kinase and its downstream target ribosomal S6 protein (rpS6) was abrogated by SNP (Fig. 4A, B).

HG-induced changes in phosphorylation of eIF4E, eIF4G, eEF2 and eEF2 kinase were also inhibited by SNP pre-treatment (Fig. 4C-F).

Since SNP is an NO donor we examined whether NO contributes to GSK3β activation by employing a structurally dissimilar NO donor. NONOate abrogated HG-induced phosphorylation of GSK3β and dephosphorylation of eIF2Bε (data not shown). Thus, SNP recruits nitric oxide (NO) pathway to mitigate high glucose-induced GSK3β Ser9 phosphorylation that results in its activation. Together these data demonstrate that HG-induced GSK3β inhibition and stimulation of events in mRNA translation are blocked by SNP thereby leading to reduced laminin synthesis in MCT cells. Based on these in vitro data, we tested if SNP can block kidney matrix protein accumulation in type 1 diabetes.

SNP administration in diabetic mice. To determine the optimal dose of SNP that activated GSK3β but did not reduce blood pressure, we administered 200, 400, and 800 µg/kg of the agent in saline daily intraperitoneally for 6 days. At 200 µg/kg dose of SNP, the blood pressure measured on alternate days was unaffected; however, at a higher dose of 800 µg/kg the blood pressure fell (Fig. 5A). At 200 µg/kg dose, there was activation of GSK3β in the kidney cortex as shown by reduction in Ser9 phosphorylation of GSK3β and increase in Ser535 phosphorylation of eIF2Bε (Fig. 5B). Thus, the 200 µg/kg/day dose of SNP was chosen and administered for 3 weeks. SNP did not affect blood glucose concentration in control or diabetic mice (Table 1). Body weight was significantly reduced and the mean arterial pressure was elevated in the diabetic mice; these parameters were unaffected by SNP (Table 1).

SNP ameliorates diabetes-induced kidney hypertrophy and albuminuria. Kidney-to-body weight ratio was increased significantly
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in diabetic mice demonstrating kidney hypertrophy (Fig. 6A), a cardinal early manifestation of kidney injury in diabetes; SNP significantly reduced renal hypertrophy (Fig. 6A), although the parameter was still higher than in non-diabetic mice (Fig. 6A). Diabetes caused an increase in albuminuria when compared to non-diabetic mice (Fig 6B); it was partially but significantly inhibited by SNP (Fig. 6B).

**SNP inhibits diabetes-induced increase in matrix proteins.** Renal tissue fibrosis due to accumulation of matrix proteins is a major contributor to kidney failure in diabetes; the pro-fibrogenic cytokine TGFβ plays an important role in stimulating renal fibrosis in diabetes (30). Diabetes increased the kidney content of fibronectin, laminin β1, and laminin γ1 by 2-3-fold that was significantly inhibited by SNP (Fig. 7A-C). Diabetic mice showed higher expression of TGFβ in the renal cortex that was associated with increase in SMAD3 phosphorylation suggesting activation of the TGFβ signaling pathway; SNP inhibited both these parameters (Fig. 7D, E). Immunoperoxidase staining for laminin trimer was performed in kidney cortical sections to assess matrix expansion (Fig. 8). Diabetes was associated with increased glomerular size and increased laminin deposition in the glomerular mesangium and, in the tubulo-interstitium when compared to kidney tissues from control mice given vehicle alone (Fig. 8C vs 8A). SNP abolished renal mesangial expansion and laminin accumulation in diabetic mice (Fig. 8D vs. 8C). Figures 8E and 8F show morphometric quantification of glomerular tuft area and fractional area of laminin staining in the glomerular tuft, respectively. Diabetes-induced significant increase in glomerular tuft area and laminin deposition was abrogated in mice treated with SNP.

**SNP inhibits diabetes-induced changes in phosphorylation of GSK3β and other signaling proteins.** We examined the signaling mechanisms involved in amelioration of kidney injury by SNP in diabetic mice. Diabetes-induced increased Ser9 phosphorylation of GSK3β was abrogated by SNP (Fig. 9A) suggesting SNP had reactivated the kinase. SNP tended to restore eIF2Bε phosphorylation in the renal cortex of diabetic mice (Fig. 9B). Interestingly, the increase in Ser21 phosphorylation in GSK3α induced by diabetes was unaffected by SNP (Fig. 9A), implying selectivity in the regulation of GSK isoforms by SNP. We explored if SNP regulated Akt and Erk, kinases that mediate HG stimulation of GSK3β phosphorylation in MCT cells (5). Diabetes increased the phosphorylation of Akt and Erk in the kidney cortex showing their activation; both parameters were partly inhibited by SNP (Fig. 9C, D). In order to identify the upstream regulators of Erk and Akt, we investigated the status of Src and Pyk2. Diabetes increased the phosphorylation of Src at Tyr416 and Pyk2 at Tyr402 that was abolished by SNP (Fig. 9E, F). These data suggest that SNP exerts its inhibitory effect on GSK3β phosphorylation by blocking the activation of its upstream kinases.

**SNP blocks diabetes-induced changes in mRNA translation.** Whether SNP activation of GSK3β in diabetes leads to inhibition of mTORC1 and downstream events in mRNA translation that participate in matrix protein synthesis was explored. Diabetes led to kidney parenchymal activation of mTORC1 as shown by increase in Thr389 and Ser240/242 phosphorylation of p70S6 kinase and ribosomal S6 protein, respectively; both the parameters were inhibited by SNP (Fig. 10A, B). In addition, diabetes-induced increase in phosphorylation of eIF4E and eEF2 kinase was also blocked by SNP (Fig. 10 C, D). Thus, SNP ameliorated diabetes-induced mTORC1 activation (Fig. 10A, B) and stimulation of initiation and elongation phases of translation (Fig 10. C, D), that are
DISCUSSION

Our findings show that in in vitro and in vivo models of diabetes-induced kidney injury, hyperglycemia leads to inhibition of GSK3β activity allowing mTORC1 activation and stimulation of events in initiation and elongation phases of mRNA translation. These effects augment synthesis of proteins including matrix proteins. Stimulation of GSK3β with SNP inhibits mTORC1 activation and protein synthesis ameliorating matrix accumulation and albuminuria in mice with type 1 diabetes.

GSK3α and β share 98% sequence identity in their catalytic domain (16). They seem to have the same substrate specificity and are thought to phosphorylate glycogen synthase at a similar rate (31). GSK3α and β appear to play a redundant role in mixed lineage proto-oncogene-driven leukemias, (32) and in maintaining the beta catenin levels in resting cells (33). Generation of mice in which phosphorylation of Ser21/9 is not possible, due to knock in of alanine residues resulting in inactivation of GSK3α and β, has shed light on functional importance of GSK3 mammalian physiology. GSK3α/β Ser21Ala, Ser9Ala knock in mice have fewer glomeruli and increased albuminuria indicating that the kinase is important for glomerular development and integrity of barrier function against proteinuria (34). Recent investigations have shown that the GSK3α and β isoforms may have distinct roles. While GSK3β knock out is embryonically lethal (35), GSK3α knock out mice are viable although they manifest accelerated aging and cardiac hypertrophy (36). An interesting finding to emerge from our studies is that GSK3β is preferably inactivated in diabetic kidney injury; SNP stimulated GSK3β rather than the GSK3α isoform, which was sufficient to ameliorate diabetic kidney injury. Additional investigation on the distinct roles of these isoforms in mediating or mitigating diabetic kidney injury will need employment of individual Ser21 and Ser9 phosphorylation deficient knock-in mice.

We examined potential upstream mechanisms that may be responsible for the protective effect of SNP on diabetes-induced kidney injury via GSK 3β activation. In addition to Akt (37), our previous studies had identified both Erk and mTOR/p70S6kinase to be upstream kinases of GSK3β in mouse proximal tubular epithelial cells (5,37). There appears to be some selectivity in upstream kinases among kidney cells because Erk and p90Rsk but not Akt phosphorylate GSK3β in renal interstitial fibroblasts upon stimulation with tissue plasminogen activator (38). SNP led to the inhibition of Erk, Akt and downstream targets of mTOR pathways in both in vitro and in vivo models employed in this study. In the present study, proximal events involved in activation of Akt and Erk in diabetes were examined in the renal cortices and in proximal tubular epithelial cells in culture. We found that diabetes activated non-receptor tyrosine kinases Src and Pyk2, which led to activation of Erk and Akt that inactivate GSK3β. Pyk2 is abundantly expressed in renal tubules; the kinase contributes to matrix accumulation in the kidney because Pyk2-/- mice have decreased renal fibrosis following ureteral obstruction (39). In contrast to our data, Src activates GSK 3β by tyrosine phosphorylation in prostate cancer (40), suggesting cell- and context-specific regulation of GSK3β by its upstream kinases. Further studies are needed to understand the mechanisms involved in diabetes-induced activation of Src and Pyk2 in the kidney.

The other factor that could lead to activation of renal Erk and Akt in diabetes is TGFβ, a fibrogenic cytokine that facilitates synthesis of general proteins and matrix
proteins contributing to renal hypertrophy and fibrosis (41). TGFβ acts via dimerization of type I and type II receptors leading to association with SMAD2, 3 and activating them by phosphorylation (42,43). Diabetic mice showed increase in TGFβ expression and stimulation of its signaling via SMAD3 in this study; SNP administration abrogated it suggesting that SNP induced reduction in Akt, Erk and mTORC1 may be in part due to inhibition of TGFβ signaling.

GSK3β regulates several cellular events including glucose metabolism and protein synthesis by phosphorylation of its substrates glycogen synthase and eIF2Bε, respectively. eIF2Bε is a guanine nucleotide exchange factor that stimulates GDP/GTP exchange reaction of eIF2 during the initiation phase of mRNA translation (44). The signaling mechanism by which SNP inhibits diabetes-induced protein synthesis appears to involve inhibition of eIF2Bε by activation of GSK3β. In addition, by inhibiting Erk activation in the kidney in diabetic mice, SNP also affected other events in initiation phase such as activation of eIF4E, the mRNA cap binding protein. Furthermore, SNP inhibited diabetes associated stimulation of mTORC1 and led to inhibition of p70S6 kinase. The latter not only facilitates the initiation phase but also directly regulates elongation phase of mRNA translation. This is achieved by inhibition of eEF2 kinase, which contributes to dephosphorylation/activation of eEF2 leading to stimulation of the elongation phase. Our data show that negative regulation of mRNA translation by GSK3β could be an important therapeutic intervention in diabetic kidney injury, since we have observed translational regulation of ECM proteins such as laminin in that disease (4,5,24,45). Together, these data show that SNP activation of GSK3β affects key regulatory steps in protein synthesis resulting in amelioration of cardinal manifestations of diabetic kidney injury.

In contrast to our current and previous finding that diabetes is associated with GSK3β inactivation in the kidney (5), others have reported stimulation of GSK3β in the diabetic kidney and amelioration associated with inactivation of the kinase (46). However, that study employed diabetic mice at a more advanced age; previous investigators have reported changes in signaling pathways in the kidneys at a longer duration of diabetes (47). GSK3β has diverse roles in kidney pathology. Activation of GSK3β is associated with apoptosis in acute tubular necrosis and administration of a single dose of lithium, a GSK3β inhibitor, is protective (48). In contrast, GSK3β seems to play a protective role in renal fibrosis associated with unilateral ureteral obstruction (49) and in a model of polycystic kidney disease (50). Thus, whether GSK3β plays an ameliorative or adverse role in kidney disease is context-specific.

The limitations of our study include the relatively short duration of observation; long-term observation is needed to explore whether regulation of GSK3β changes with time. Our data do not address the efficacy of SNP in ameliorating kidney injury in a model of type 2 diabetes. Additional work is needed to explore if kidney inflammation is affected by stimulation of GSK3β in diabetic mice and if agents that only stimulate GSK3β would be as beneficial as SNP, which also affected the upstream kinases Akt and Erk. Future studies are planned to address these issues. In conclusion, our results suggest that inhibition of GSK3β contributes to kidney injury during the initial stages of diabetes. It provides evidence that GSK3β/eIF2Bε axis may be a therapeutic target to reduce renal ECM protein accumulation in diabetes.
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FOOTNOTES

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The abbreviations used are: SNP-sodium nitroprusside, MCT cells- proximal tubular epithelial cells, HG-high glucose.

Table Legend

**Table 1. Clinical Parameters.** Blood glucose, body weight and blood pressure in type 1 diabetic mice administered sodium nitroprusside for 3 weeks. The results are represented as mean±SD for 4 to 8 mice in each group. P<0.05 is considered to be significant change.
**Figure Legends**

**FIGURE 1. Sodium nitroprusside (SNP) activates GSK3β in proximal tubular epithelial (MCT) cells.** (A) Equal amounts of cell lysate protein were immunoblotted with specific antibodies to detect high glucose (HG, 30 mM)-induced changes in phosphorylation of GSK 3α/β at Ser21/9 and at Tyr279/216 and eIF2Bε at Ser535. (B) Dose-dependent effect of SNP on phosphorylation of GSK3β at Ser9 and eIF2Bε at Ser535 in MCT cells. (C) Effect of SNP on HG-induced changes in phosphorylation of GSK3β on Ser9 and eIF2Bε on Ser535. Loading of equal protein was assessed by immunoblotting using antibodies against respective proteins. Representative blots and histogram of composite data from 3 to 4 experiments are shown in Panels A-C.

**FIGURE 2. Sodium nitroprusside (SNP) inhibits high glucose stimulation of matrix protein expression in MCT cells.** MCT cells were treated with or without high glucose for 15 min following pre-incubation with or without SNP for 30 minutes. Equal amounts of cell lysate protein were immunoblotted with specific antibodies to detect changes in (A) laminin β1, (B) laminin γ1. Immunoblotting showed that SNP blocked high glucose-induced phosphorylation and activation of (C) Akt and (D) Erk. Loading of equal protein was assessed by immunoblotting using antibodies against respective proteins. Representative blots and histogram of composite data from 3 to 4 experiments are shown.

**Figure 3. High glucose-induced activation of Src and Pyk2 is required for matrix protein synthesis via GSK 3β inactivation.** Equal amounts of cell lysate protein were immunoblotted with respective antibodies. Pre-incubation of cells with SNP inhibited HG-induced activation of Src (A) and Pyk2 (B). Pre-incubation with PP2 (a Src inhibitor) and BAPTA/AM (a Pyk2 inhibitor) blocked HG-induced GSK3β phosphorylation at Ser9 (3C and 3D) and synthesis of laminin γ1 (3E and 3F). HG-induced activation of Akt and Erk was blocked by PP2 (3G and 3I) and BAPTA/AM (3H and 3J). Immunoblotting using antibody against the respective total proteins or actin was done to assess loading. Representative blots and histogram of composite data from 3 to 4 experiments are shown.

**FIGURE 4. Sodium nitroprusside inhibits high glucose-induced events in mRNA translation in MCT cells.** MCT cells were treated with or without high glucose following pre-incubation with or without SNP for 15 minutes. Equal amounts of cell lysate protein were immunoblotted with specific antibodies to detect changes in phosphorylation status of (A) p70S6 kinase, (B) ribosomal S6 proteins, (C) eIF4E, (D) eIF4G, (E) eEF2 and (F) eEF2 kinase. Loading of equal protein was assessed by immunoblotting using antibodies against respective proteins. Representative blots and histogram of composite data from 3 to 4 experiments are shown.

**FIGURE 5. Determination of optimal dose of sodium nitroprusside (SNP).** (A). Mice were injected intra-peritoneally with saline or sodium nitroprusside (SNP) 200 µg/kg, 400 µg/kg and 800 µg/kg in a similar volume daily for 6 days. Blood pressure was measured on alternate days by the tail cuff method in conscious mice using the CODA non-invasive blood pressure system (Kent Scientific Corporation, Torrington, CT). Mice were trained for 1 week on the restrainer placed on a warm platform. Blood pressure (systolic, diastolic and mean arterial pressure) in each mouse was recorded over 30 cuff inflations after 10 training cuff inflations (51). (B) Mice
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treated as above were sacrificed on day 6. Renal cortical homogenates were immunoblotted with antibodies against phospho-Ser9 GSK3β, phospho-Ser535 eIF2Be, GSK 3β or eIF2Be. The histogram shows quantitative data from 3 mice in each group.

FIGURE 6. Sodium nitroprusside ameliorates albuminuria and kidney hypertrophy in diabetic mice. SNP or vehicle was administered to control (Con) and STZ-induced type 1 diabetic (Diab) mice for 3 weeks as described in the methods section. SNP ameliorated (A) kidney hypertrophy (n= 5-8 in each group) and (B) albuminuria (n= 5-8 in each group).

FIGURE 7. Sodium nitroprusside inhibits kidney matrix protein increment in diabetic mice. Equal amounts of renal cortical lysate protein were immunoblotted with specific antibodies for matrix proteins (A) Fibronectin, (B) Laminin β1 and (C) Laminin γ1. Panels D and E show regulation of TGFβ expression and SMAD3 phosphorylation, respectively. Loading was assessed by immunoblotting for actin or SMAD3. Representative blots and histograms are shown for n= 5-8 mice per group.

FIGURE 8. Immunohistochemical analysis of renal laminin expression. Immunoperoxidase staining with an antibody against laminin trimer showed increased deposition in glomerular mesangium in kidneys from diabetic mice (C) compared to non-diabetic control mice (A). SNP (D) ameliorated diabetes-induced changes in renal laminin expression when compared to kidney from diabetic mice (C). The histogram shows composite averages of renal glomerular tuft area (E) and immunoperoxidase staining intensities of laminin (F) in control and diabetic mice treated with or without SNP. For each mouse, 25 to 40 glomeruli were analyzed (n=4 in each group).

FIGURE 9. Sodium nitroprusside inhibits diabetes-induced signaling reactions in the kidney cortex. Equal amounts of protein from renal cortical lysate were immunoblotted with corresponding antibodies to assess changes in phosphorylation of (A) GSK 3α/β and eIF2Be (B). Immunoblotting was done to identify upstream regulators of GSK 3β phosphorylation: (C) Akt, (D) Erk, (E) Src, and, (F) Pyk2. Immunoblotting with antibodies against respective total protein was done to assess loading. Representative blots and histograms are shown for 5 to 8 mice in each group.

FIGURE 10. Sodium nitroprusside inhibits diabetes-induced events in mRNA translation in kidney cortex. Equal amounts of renal cortical lysate protein were immunoblotted with antibodies to assess changes in phosphorylation of (A) p70S6 kinase (B) ribosomal S6 protein, (C) eIF4E, and, (D) eEF2 kinase. Loading was assessed by immunoblotting with antibodies for respective total protein. Representative blots and histograms are shown for 4 to 7 mice in each group.
**Table 1. Clinical parameters.**  
The results are represented as mean ± SD; n= 4 to 8 mice in each group. (Con=control, Veh=Vehicle, SNP=Sodium nitroprusside, MAP= mean arterial pressure). *p<0.001 vs Con+Veh.

|                  | Con+Veh  | Con+SNP  | Diab    | Diab+SNP |
|------------------|----------|----------|---------|----------|
| Blood glucose (mg/dL) | 132±20   | 148 ±10  | 381 ±63*| 391 ±45* |
| Body Wt (g)      | 26.03±2.42| 26.55 ±1.56| 16.83 ±0.90*| 16.10 ±1.48* |
| MAP (mm Hg)      | 100.13 ±9.36| 105.09 ±11.29| 139.82 ±16.38*| 140.13 ±13.05* |
Figure 1

A

30mM glucose (min) 0 10 15 30 60 120

P-GSK 3α/β Ser 21/9

P-GSK 3 α/β Tyr 279/216

GSK 3 α/β

P-eIF2Be Ser535

eIF2Be

B

SNP (µM) 15 min

P GSK 3β Ser9

GSK 3β

P eIF2Be Ser535

eIF2Be

C

SNP (50µM) – + – +

Glucose (30mM) – – + +

P GSK 3β Ser 9

GSK 3β

P-eIF2Be Ser535

eIF2Be
Figure 2

A

SNP (50uM)  −  +  −  +
Glucose (30mM)  −  −  +  +
Laminin β1
Actin

p<0.05  p<0.05

B

SNP (50uM)  −  +  −  +
Glucose (30mM)  −  −  +  +
Laminin γ1
Actin

p<0.05  p<0.05

C

SNP (50uM)  −  +  −  +
Glucose (30mM)  −  −  +  +
P Akt
Akt

p<0.001  p<0.001

D

SNP (50uM)  −  +  −  +
Glucose (30mM)  −  −  +  +
P Erk
Erk

p<0.05  p<0.01
Figure 3

A

SNP (50uM)  
Glucose (30mM)  

P Src  
Src  

p<0.05  p<0.05

P Src/Src  
Arbitrary units

B

SNP (50uM)  
Glucose (30mM)  

P Pyk2  
Pyk2  

p<0.05  p<0.05

P Pyk2/Pyk2  
Arbitrary units

C

PP2 (20uM)  
Glucose (30mM)  

P GSK 3b  
GSK3b  

p<0.05  p<0.01

P GSK 3b/GSK 3\beta  
Arbitrary units

D

BAPTA/AM (100uM)  
Glucose (30mM)  

P GSK 3b  
GSK3b  

p<0.01  p<0.001

P GSK 3b/GSK 3\beta  
Arbitrary units

E

PP2 (20uM)  
Laminin γ1  

Actin  

p<0.001  p<0.01

Laminin γ1/Actin  
Arbitrary units

F

BAPTA/AM (100uM)  
Glucose (30mM)  

P GSK 3b  
GSK3b  

p<0.01  p<0.001

P GSK 3b/GSK 3\beta  
Arbitrary units

G

PP2 (20uM)  
Glucose (30mM)  

P Akt  
Akt  

p<0.001  p<0.001

P Akt/Akt  
Arbitrary units

H

BAPTA/AM (100uM)  
Glucose (30mM)  

P Akt  
Akt  

p<0.01  p<0.05

P Akt/Akt  
Arbitrary units

I

PP2 (20uM)  
Glucose (30mM)  

P Erk  
Erk  

p<0.05  p<0.05

P Erk/Erk  
Arbitrary units

J

BAPTA/AM (100uM)  
Glucose (30mM)  

P Erk  
Erk  

P<0.01  p<0.05

P Erk/Erk  
Arbitrary units
Figure 4

A

SNP (50uM)  
Glucose (30mM)  

P p70S6K  
p70S6K

p<0.05  p<0.05

P p70S6K/p70S6K
Arbitrary units

B

SNP (50uM)  
Glucose (30mM)  

P rpS6  
 rpS6

p<0.05  p<0.05

P rpS6/rpS6
Arbitrary units

C

SNP (50uM)  
Glucose (30mM)  

P eIF4E  
eIF4E

p<0.01  p<0.05

P eIF4E/eIF4E
Arbitrary units

D

SNP (50uM)  
Glucose (30mM)  

P eIF4G  
eIF4G

p<0.01  p<0.05

P eIF4G/eIF4G
Arbitrary units

E

SNP (50uM)  
Glucose (30mM)  

P eEF2  
eEF2

p<0.01  p<0.05

P eEF2/eEF2
Arbitrary units

F

SNP (50uM)  
Glucose (30mM)  

P eEF2K  
eEF2K

p<0.01  p<0.05

P eEF2K/eEF2K
Arbitrary units
Figure 5

A

| Groups          | Day 2        | Day 4        | Day 6        | Avg. MAP     |
|-----------------|--------------|--------------|--------------|--------------|
| Saline          | 113.88±20.96 | 101.53±12.97 | 109.81±0.91  | 108.40±10.09 |
| SNP 200μg/kg    | 100.80±2.19  | 102.7±8.28   | 108.16±7.22  | 102.83±8.23  |
| SNP 400μg/kg    | 118.32±2.78  | 101.24±8.73  | 96.70±7.03   | 106.36±3.06  |
| SNP 800μg/kg    | 87.97±8.7    | 88.87±4.27   | 81.27±6.31   | 85.71±2.26   |

B

[Graph showing changes in P GSK 3β/GSK 3β and P eIF2Bε/eIF2Bε with concentration of SNP (μg/kg).]

- *p* < 0.05
- *p* < 0.01
- *p* < 0.01

Concentration of SNP (μg/kg)
Figure 6

A

Kidney Wt./Body Wt. (mg/g)

% of control

Con Con+SNP Diab SNP+Diab

p<0.001

p<0.01

B

Alb:Creatinine ratio (mg/mg)

Con Con+SNP Diab SNP+Diab

p<0.01

p<0.05
Figure 7

A  

|          | Control | Con+SNP | Diab | Diab+SNP |
|----------|---------|---------|------|----------|
| Fibronectin |  
| Actin |  

B  

|          | Control | Con+SNP | Diab | Diab+SNP |
|----------|---------|---------|------|----------|
| Laminin β1 |  
| Actin |  

C  

|          | Control | Con+SNP | Diab | Diab+SNP |
|----------|---------|---------|------|----------|
| Laminin γ1 |  
| Actin |  

D  

|          | Control | Con+SNP | Diab | Diab+SNP |
|----------|---------|---------|------|----------|
| TGF β |  
| Actin |  

E  

|          | Control | Con+SNP | Diab | Diab+SNP |
|----------|---------|---------|------|----------|
| P SMAD3 |  
| SMAD3 |  

**A**  

- Control: p<0.05  
- Con+SNP: p<0.01  
- Diab: p<0.01  
- Diab+SNP: p<0.01  

**B**  

- Control: p<0.05  
- Con+SNP: p<0.01  
- Diab: p<0.05  
- Diab+SNP: p<0.05  

**C**  

- Control: p<0.05  
- Con+SNP: p<0.01  
- Diab: p<0.01  
- Diab+SNP: p<0.01  

**D**  

- Control: p<0.05  
- Con+SNP: p<0.01  
- Diab: p<0.05  
- Diab+SNP: p<0.05  

**E**  

- Control: p<0.05  
- Con+SNP: p<0.01  
- Diab: p<0.01  
- Diab+SNP: p<0.05
Figure 8

A. Control

B. Con+SNP

C. Diab

D. Diab+SNP

E. Glomerular tuft area $\times 10^3 \mu m^2$

F. Mesangial Laminin fraction % of glomerular area

p<0.01    p<0.01    p<0.01    p<0.05
Figure 9

A

| Control | Con+SNP | Diab | Diab+SNP |
|---------|---------|------|----------|
| P.GSK3α/β | α | β | p<0.05 | p<0.01 | p<0.001 |

B

| Control | Con+SNP | Diab | Diab+SNP |
|---------|---------|------|----------|
| P.eIF2Be | eIF2Be | p<0.05 | | |

C

| Control | Con+SNP | Diab | Diab+SNP |
|---------|---------|------|----------|
| P.Akt | Akt | p<0.001 | p<0.05 |

D

| Control | Con+SNP | Diab | Diab+SNP |
|---------|---------|------|----------|
| P.Erk | Erk | p<0.01 | p<0.05 |

E

| Control | Con+SNP | Diab | Diab+SNP |
|---------|---------|------|----------|
| P.Src | Src | p<0.001 | p<0.001 |

F

| Control | Con+SNP | Diab | Diab+SNP |
|---------|---------|------|----------|
| P.Pyk2 | Pyk2 | p<0.001 | p<0.01 |
Figure 10

A

|        | Control | Con+SNP | Diab | Diab+SNP |
|--------|---------|---------|------|----------|
| N=5    |         |         |      |          |
| N=6    |         |         |      |          |
| N=8    |         |         |      |          |
| N=7    |         |         |      |          |

P.p70S6K

p70S6K

B

|        | Control | Con+SNP | Diab | Diab+SNP |
|--------|---------|---------|------|----------|
| N=5    |         |         |      |          |
| N=6    |         |         |      |          |
| N=8    |         |         |      |          |
| N=7    |         |         |      |          |

P. rpS6

rpS6

C

|        | Control | Con+SNP | Diab | Diab+SNP |
|--------|---------|---------|------|----------|
| N=5    |         |         |      |          |
| N=6    |         |         |      |          |
| N=8    |         |         |      |          |
| N=7    |         |         |      |          |

P.eIF4E

eIF4E

D

|        | Control | Con+SNP | Diab | Diab+SNP |
|--------|---------|---------|------|----------|
| N=5    |         |         |      |          |
| N=6    |         |         |      |          |
| N=8    |         |         |      |          |
| N=7    |         |         |      |          |

P.eEF2K

eEF2K

Bar graphs show the fold change in protein expression levels for each condition. The p-values indicate statistical significance between the groups.
Activation of Glycogen Synthase Kinase 3β Ameliorates Diabetes-Induced Kidney Injury
Meenalakshmi M. Mariappan, Sanjay Prasad, Kristin D'Silva, Esteban Cedillo, Kavithalakshmi Sataranatarajan, Jeffrey L. Barnes, Goutam Ghosh Choudhury and Balakuntalam S. Kasinath

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