Wld<sup>S</sup> ameliorates renal injury in a type 1 diabetic mouse model

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Zhu S, Yang Y, Hu J, Qian L, Jiang Y, Li X, Yang Q, Bai H, Chen Q. Wld<sup>S</sup> ameliorates renal injury in a type 1 diabetic mouse model. Am J Physiol Renal Physiol 306: F1348–F1356, 2014. First published March 5, 2014; doi:10.1152/ajprenal.00418.2013.—Diabetic nephropathy (DN) is the leading cause of end-stage kidney disease worldwide. The purpose of this study is to investigate whether the Wld<sup>S</sup> (slow Wallerian degeneration; also known as Wld) gene plays a renoprotective role during the progression of DN. Diabetes was induced in 8-wk-old male wild-type (WT) and C57BL/Wld<sup>S</sup> mice by streptozotocin (STZ) injection. Blood and urinary variables including blood glucose, glycated hemoglobin (GHb), insulin, urea nitrogen, and albumin/creatinine ratio were assessed 4, 7, and 14 wk after STZ injection. Periodic acid-Schiff staining, Masson staining, and silver staining were performed for renal pathological analyses. In addition, the renal ultrastructure was observed by electron microscope. The activities of p38 and ERK signaling in renal cortical tissues were evaluated by Western blotting. NAD<sup>+</sup>/NADH ratio and NADPH oxidase activity were also measured. Moreover, the expressions of TNF-α, IL-1, and IL-6 were examined. We provide experimental evidence demonstrating that the Wld<sup>S</sup> gene is expressed in kidney cells and protects against the early stage of diabetes-induced renal dysfunction and extracellular matrix accumulation through delaying the reduction of the NAD<sup>+</sup>/NADH ratio, inhibiting the activation of p38 and ERK signaling, and suppressing oxidative stress as evidenced by the decreased NADPH oxidase activity and lower expression of TNF-α, IL-1, and IL-6.

Wld<sup>S</sup>, diabetic renal injury; oxidase stress; NAD<sup>+</sup>/NADH ratio; p38 and ERK

DIABETIC NEPHROPATHY (DN) IS ONE OF THE MOST IMPORTANT COMPLICATIONS OF DIABETES PATIENTS AND LEADS TO END-STAGE RENAL FAILURE; ~30% OF TYPE 1 DIABETIC PATIENTS SUFFER FROM DN (10, 29). IT FEATURES HISTOLOGICALLY BY RENAL HYPERPOTHEPSY, EXTRACELLULAR MATRIX ACCUMULATION, AND THE THICKENING OF THE GLOMERULAR BASEMENT MEMBRANE (GBM). THE PATHOGENESIS OF DN IS MULTIFACTORIAL AND INVOLVES ACTIVATION OF SEVERAL PATHWAYS LEADING TO KIDNEY DAMAGE, INCLUDING THE POLYSOMAL PATHWAY, ADVANCED GLYCATION END PRODUCTS, OXIDATIVE STRESS, PROINFLAMMATORY CYTOKINES, AND PROBROBIOTIC GROWTH FACTORS (30, 42). HYPERGLYCEMIA TRIGERS A SERIES OF INTRACELLULAR EVENTS IN GLOMERULAR AND TUBULAR CELLS SUCH AS REACTIVE OXYGEN SPECIES (ROS) GENERATION, PKC AND MAPK ACTIVATION, AND TRANSCRIPTION FACTOR INDUCTION (21, 24, 27, 35). DOWNSTREAM MEDIATORS OF OXIDANT INJURY INCLUDE POLY(ADP-RIBOSE) POLYMERASE (PARP). ACTIVATION OF PARP CONTRIBUTES TO NAD<sup>+</sup> DEPLETION AND ENERGY FAILURE AND IS IMPlicated IN MULTIPLE CHANGES CHARACTERISTIC OF EARLY NEPHROPATHY ASSOCIATED WITH TYPE 1 DIABETES (9, 16). Wld<sup>S</sup>, a chimeric protein from a spontaneous mutation containing full-length nicotinamide mononucleotide adenyllytransferase 1, has NAD biosynthesis activity (20, 34, 49). It attenuates the axon degeneration that is associated with several neurodegenerative disease models (13, 18, 19, 36, 38). In our previous study, we have shown that Wld<sup>S</sup> could attenuate experimental diabetes and early experimental peripheral diabetic neuropathy and diabetic retinopathy (52). Here, we wanted to determine whether the Wld<sup>S</sup> gene plays a renoprotective role during the progression of diabetic renal injury.

MATERIALS AND METHODS

Animal model. Homozygous C57BL/Wld<sup>S</sup> mutant mice were obtained from the Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences and Chinese Academy of Sciences. In all studies, C57BL/Wld<sup>S</sup> mice were genotyped 4 wk after birth. Eight-week-old male C57BL/6J wild-type (WT) mice and homozygous C57BL/Wld<sup>S</sup> mice were housed under a 12:12-h light-dark cycle in pathogen-free conditions with free access to mouse chow and water. Diabetes was induced by injecting mice intraperitoneally with 150 mg/kg body wt of streptozotocin (STZ; Sigma, St Louis, MO) dissolved in citrate buffer (pH 5.5). Nondiabetic mice were injected with sodium citrate buffer alone. Animal fasting weight and tail vein blood glucose concentrations were measured 1 wk after STZ administration and every other week using a OneTouch Horizon Glucose Monitoring Kit (LifeScan, Milpitas, CA). Only STZ-injected mice with fasting blood glucose concentrations greater than or equal to 11.1 mmol/l were included in the diabetic groups. Systolic blood pressure was measured at 14 wk after STZ injection using tail-cuff plethysmography (BP-2000; Visitech Systems, Apex, NC). All animal care and use were in accordance with guidelines established by the Research Animal Care Committee of Nanjing Medical University.

Metabolic data. At 4, 7, and 14 wk after injection, urine collection was performed for 24 h, with each mouse individually housed in a metabolic cage with free access to food and water. Then, mice were euthanized, and blood and kidney tissue were harvested. Part of the blood was collected in a heparinized tube, and plasma was separated by centrifugation at 2,000 rpm for 30 min. The other portion of the blood was collected in a nonheparinized tube, and serum was separated by centrifugation at 3,000 rpm for 15 min. GHb was measured using a VARIANT II Hemoglobin Analyzer (Bio-Rad, Hercules, CA). Plasma blood urea nitrogen (BUN), urinary creatinine, and urinary albumin were measured using an AU2700 Automated Chemistry Analyzer (Olympus, Tokyo, Japan). Serum insulin was measured with a radioimmunoassay kit (Bnibt, Beijing, China). Both kidneys were removed and weighed to evaluate the kidney weight-to-body weight ratio. The left kidney was fixed in 10% buffered formalin followed by embedding in paraffin. The right kidney was flash frozen in liquid nitrogen and stored at −80°C for Western blot analysis and measurement of NAD<sup>+</sup>/NADH ratio and NADPH oxidase activity.

Renal morphology assessment. Tissue sections from paraffin-embedded kidneys were stained with periodic acid-Schiff (PAS), Masson’s trichrome, and hematoxylin and eosin. The examination of slides was performed under light microscopy with magnifications of ×400 and ×1,000. The surface area (µm<sup>2</sup>) of a minimum of 25 individual glomerular sections from 6 individual mice was determined in digital
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images using Image Pro Plus 5.0 software. The areas that stained positively with PAS within the glomerular tuft were counted. The mesangial matrix index (%) was expressed as mesangial matrix area/tuft area × 100%.

Electron microscopic evaluation. For studying renal ultrastructure, kidney tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) overnight at 4°C. Tissues were washed three times for 15 min with 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h. After a washing three times for 15 min with 0.1 M phosphate buffer, tissues were embedded in Epon following dehydration in a graded series of ethanol preparations. Ultrathin sections (60 nm), which were poststained with lead citrate and uranyl acetate, were prepared and then examined using a JEOL-1010 microscope (JEOL, Tokyo, Japan) with magnifications of ×8,000 and ×30,000.

RNA extraction, RT, and gene expression. mRNAs of frozen kidneys and cerebellums were extracted using RNAiso Plus (Takara, Dalian, China). mRNA was transformed into cDNA using a Prime Script RT reagent kit (Takara); RT-PCR was performed to identify WNDs gene expression. The following primer sequences were used: phospho-p38, total p38, phosho-ERK and total ERK (Cell Signal, Boston, MA), and α-tubulin (Millipore, Billerica, MA). Detection was performed using horseradish peroxidase-labeled anti-rabbit IgG or horseradish peroxidase-labeled anti-mouse IgG and developed with Super-Signal chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). NAD+/NADH assay. Frozen kidneys were extracted by two freeze-thaw cycles or homogenization in 400 μl NAD+/NADH Extraction Buffer (BioVision, Mountain View, CA). The homogenate was filtered using 10-kDa cut-off filters. The assay was conducted following the manufacturer’s instructions.

NADPH oxidase activity. Renal NADPH oxidase activity, expressed as the NADP/NADPH ratio, was measured with a commercial kit (Genmed Scientific, Arlington, MA) by reading absorbance at 550 nm on a microplate reader. The greater the optical density, the higher the NADPH oxidase activity was determined to be.

Statistical analysis. Statistical analysis was conducted using GraphPad Prism Software version 5.0. Results are presented as means ± SE. Differences were determined using a two-tailed Student’s t-test or ANOVA, with a Newman-Keuls test to determine post hoc differences. Differences were considered significant at a two-tailed P value of <0.05.

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Fig. 1. Expression of Wndt in Wndt mouse kidneys was identified by RT-PCR (A) and Western blotting (B). The RT-PCR product amplified and protein extracted from Wndt mouse cerebellum were used as a positive control individually.

Fig. 2. Fasting blood glucose, serum insulin, and GHb levels in mice with a single high dose of streptozotocin (STZ; A) Fasting tail vein blood glucose concentrations were measured 1 wk after STZ injection and every other week (B and C). Fasting serum insulin and GHb concentrations were measured 4, 7, and 14 wk after induction of diabetes. Values are means ± SE; n = 5–20/group: wild type (WT) + STZ, STZ-induced diabetic WT mice; Wndt + STZ, STZ-induced diabetic Wndt mice. *P < 0.05 compared with control group.
RESULTS

Wld⁶ protein is highly expressed in the kidney and improves metabolic disorders in a STZ-induced diabetic renal injury model. Through RT-PCR and Western blotting, we confirmed that Wld⁶ was highly expressed in the kidney (Fig. 1). To assess the effects of Wld⁶ on the development of diabetic renal injury, we treated the Wld⁶ and WT mice with one single high dose of STZ (150 mg/kg) to induce diabetes. After STZ injection, blood glucose levels were tested and only those with blood glucose levels greater than or equal to 11.1 mmol/l were considered to be diabetic. Both the STZ-treated and control mice were further maintained for 14 wk under normal rearing conditions to allow the development of diabetic kidney diseases. In addition, all the diabetic mice had similar fasting blood glucose and serum insulin concentrations (Fig. 2, A and B) as well as GHb levels (Fig. 2C). Four weeks after diabetes onset, the kidney weights per body weight of the diabetic groups were significantly higher than those of the nondiabetic groups. Wld⁶ appeared to result in significant improvement on the ratio of kidney weight to body weight at 7 and 14 wk after diabetes onset (Fig. 3A). BUN and the urinary albumin/creatinine ratio kept rising during these 14 wk in the diabetic WT group, and Wld⁶ can significantly inhibit growth in the diabetic group. Systolic blood pressure was similar in all groups (Fig. 3, B–D).

Diabetic Wld⁶ mice show fewer morphological signs of nephropathy. To examine potential renal cortical morphological differences in the diabetic WT mice and diabetic Wld⁶ mice, we performed PAS staining, Masson’s trichrome, and silver staining with the renal cortical tissues dissected from six mice per group. As shown in Fig. 4, no apparent difference in glomerular morphology was observed for nondiabetic WT and Wld⁶ mice. The glomeruli from both groups were of normal size and configuration. Furthermore, there was no sign of mesangial matrix expansion, inflammation, or sclerosis in these mice. In diabetic WT mice, there was a significant increase in nephropathy compared with control mice after 4 wk, the glomerular surface area increased, and the mesangium was diffusively and markedly expanded with PAS-positive (purple color) matrix material. However, in diabetic Wld⁶ mice, less glomerular hypertrophy and matrix accumulation around the capillaries of the glomeruli were observed (Figs. 4 and 8A). Masson’s trichrome stain indicated prominent elevated levels of collagen deposition in the glomeruli of diabetic WT mice, whereas the interstitial fibrosis was mild in diabetic Wld⁶ mice (Fig. 5). Furthermore, silver staining showed the GBMs of diabetic WT mice were thicker than control mice, whereas this change was also mild in diabetic Wld⁶ mice (Fig. 6). In addition, electron microscopy revealed that diabetic WT mice, especially the 14-wk group, showed increased mesangial matrix, evident extensive fusion of foot processes, and thicker GBMs. In contrast, diabetic Wld⁶ mice showed much fewer of these abnormal alterations (Fig. 7).

Wld⁶ protein appears to ameliorate diabetes-induced reduction of the NAD⁺/NADH ratio, oxidative stress, and activation of p38 and ERK signaling. Next, we explored how Wld⁶ exerts its function. The NAD⁺/NADH ratio was evaluated at 4, 7, and 14 wk after diabetes onset. In diabetic WT mice, the NAD⁺/NADH ratio was dramatically decreased at 7 wk, and Wld⁶ can ameliorate this reduction. At 14 wk, the NAD⁺/NADH ratio of

![Fig. 3. A: kidney/body weight ratios were all higher in diabetic mice than control mice, which was prevented by Wld⁶ in diabetic mice at 7 and 14 wk after diabetes onset. Blood urea nitrogen (BUN; B) and urinary albumin/creatinine ratio (C) were sharply increased in diabetic WT mice, which was significantly inhibited by Wld⁶ in diabetic mice. Systolic blood pressure (D) was similar in all control and diabetic mice. Values are means ± SE; n = 5–12/group. Groups are as defined in Fig. 2. *P < 0.05 compared with control group. **P < 0.05 compared with STZ-treated WT mice.](http://ajprenal.physiology.org/Downloaded from)
diabetic WldS mice tended to be higher compared with diabetic WT mice, but the difference was not significant (Fig. 8B). Moreover, WldS markedly suppressed the enhanced NADPH oxidase activity in kidneys from diabetic mice (Fig. 8C). To investigate the role of inflammatory mediators on WldS-mediated nephroprotective effect, mRNA expression levels of TNF-α, IL-1, and IL-6 were determined using qRT-PCR. Figure 9 showed that WldS significantly inhibited the diabetes-mediated induction of TNF-α, IL-1, and IL-6 mRNA levels. It is well known that the activation of MAPK pathways, especially p38 and ERK pathways, plays an important role in DN (5, 15, 48). Therefore, we assayed the effect of WldS on the activation of p38 and ERK signaling pathways by Western blot.

Fig. 4. Mesangial matrix expansion in four groups. At 4, 7, and 14 wk after the onset of diabetes, renal sections were stained with periodic-acid-Schiff (PAS). Results are representative of samples from each group (n = 6/group). Less matrix accumulation was shown in diabetic WldS mice. A: original magnification, ×400; scale bar = 20 μm. B: original magnification, ×1,000; scale bar = 5 μm.

Fig. 5. Collagen deposition in 4 groups. At 4, 7, and 14 wk after the onset of diabetes, renal sections were stained with Masson’s trichrome. Results are representative of samples from each group (n = 6/group). Less interstitial fibrosis was shown in diabetic WldS mice. A: original magnification, ×400; scale bar = 20 μm. B: original magnification, ×1,000; scale bar = 5 μm.
analysis. As shown in Fig. 10, STZ injection resulted in significantly increased phosphorylation of p38 and ERK at 4 and 7 wk, and these increases were remarkably attenuated by WldS in diabetic mice. At 14 wk, the increase in phospho-p38 was also reduced by WldS, but there is no significant difference in p-ERK between WT and WldS diabetic mice.

DISCUSSION

In the current study, we demonstrated that WldS improved common metabolic disorder indices associated with diabetic renal injury and ameliorated the pathological characteristics, including renal hypertrophy, extracellular matrix deposition, thickening of basement membranes, and foot process fusion. Additionally, WldS effectively alleviated the reduction of the NAD+/NADH ratio, increased NADPH oxidase activity, up-regulated expression of inflammatory cytokines, and the activation of p38 and ERK signaling pathways in renal tissues of diabetic mice, suggesting the possible mechanisms by which WldS exerts its renoprotective effects on diabetic mice. To our knowledge, this is the first report investigating the renoprotective effects of WldS on diabetic renal injury.

Oxidative stress has been implicated in the pathogenesis of DN. High glucose induces intracellular ROS in glomerular mesangial and tubular epithelial cells. PKC, NAD(P)H oxi-
P/H11021 treated WT mice. NADPH oxidase activity in kidneys from control and diabetic mice. WldS (INO-1001, PJ34, 1,5-isoquinolinediol, and GPI-15,427) and induced by ROS-trigged PARP activation. PARP inhibition that a NMNAT-containing protein such as WldS may also stress in the kidney (2, 4, 11, 14, 27, 44). Our results revealed oxidase inhibition prevented the diabetes-induced oxidative tubules of DN animals (12, 44). Direct or indirect NADPH kidney cortex, and mesangial cells (8, 17, 26). NAD(P)H is a major source of ROS in renal cells such as glomerular glucose-induced ROS generation (32, 39). NAD(P)H oxidase, and mitochondrial metabolism all play roles in high NAD retard diabetic renal injury development by increasing the NAD+/NADH ratio and inhibiting the increase in NADPH oxidase activity.

P/H11001 WT +ST Z WdlS WldS+STZ WldS+STZ WT +ST Z WT

WldS ameliorated hypertrophy in diabetic mice at 4 and 7 wk after diabetes onset. B: NAD+/NADH ratio in kidneys from control and diabetic mice. WldS delayed the reduction of the NAD+/NADH ratio in diabetic mice. C: NADPH oxidase activity in kidneys from control and diabetic mice. WldS reduced the increase in NADPH oxidase activity in kidneys from diabetic mice. Values are means ± SE; n = 6/group. Groups are defined as in Fig. 2. *P < 0.05 compared with control group. #P < 0.05 compared with STZ-treated WT mice.

dase, and mitochondrial metabolism all play roles in high glucose-induced ROS generation (32, 39). NAD(P)H oxidase is a major source of ROS in renal cells such as glomerular mesangial and tubular epithelial cells. As a catalytic subunit of NAD(P)H oxidases, Nox4 is abundant in the vascular system, kidney cortex, and mesangial cells (8, 17, 26). NAD(P)H oxidase expression is upregulated in the glomerulus and distal tubules of DN animals (12, 44). Direct or indirect NADPH oxidase inhibition prevented the diabetes-induced oxidative stress in the kidney (2, 4, 11, 14, 27, 44). Our results revealed that a NMNAT-containing protein such as WldS may also retard diabetic renal injury development by increasing the NAD+/NADH ratio and inhibiting the increase in NADPH oxidase activity.

There is a decrease in the NAD+/NADH ratio in renal tissues of DN. NAD+ depletion and energy failure can be induced by ROS-trigged PARP activation. PARP inhibition (INO-1001, PJ34, 1,5-isoquinolinediol, and GPI-15,427) and PARP-1 gene deficiency are examples of nephroprotection (9, 40, 41, 43). Moreover, a higher NAD or NAD+/NADH ratio can activate the sirtuin 1 (SIRT1) pathway (3, 47), which is a known renal cytoprotector responding to aging and stress (51). Many reagents exert nephroprotective effects by promoting NAD+ production. For example, resveratrol (trans-3,4',5-tri-hydroxyestilbene; RSV) ameliorates renal injury and promotes mitochondrial biogenesis through inhibiting oxidative stress, which is mediated via the AMPK/SIRT1-independent pathway (6, 31, 50). Rapamycin exhibits an inhibitive effect on glucose uptake and utilization in mesangial cells, which leads to the increase in NAD+/NADH levels and upregulation of SIRT1 (51). Furthermore, nicotinamide (vitamin B3), a major NAD+ precursor, is also shown to have a protective effect against nephropathy in diabetic rats (46). One randomized trial of participants with DN and stages 1-3 chronic kidney disease showed that the use of high doses of B vitamins (containing 2.5 mg/day of folic acid, 25 mg/day of vitamin B6, and 1 mg/day of vitamin B12) resulted in a greater decrease in glomerular filtration rate compared with placebo (23). WldS is an intrinsic

Fig. 8. A: quantitative analyses of glomerular sizes in 4 groups of mice. Glomerular surface area was measured as described in the MATERIALS AND METHODS. WldS ameliorated hypertrophy in diabetic mice at 4 and 7 wk after diabetes onset. B: NAD+/NADH ratio in kidneys from control and diabetic mice. WldS delayed the reduction of the NAD+/NADH ratio in diabetic mice. C: NADPH oxidase activity in kidneys from control and diabetic mice. WldS reduced the increase in NADPH oxidase activity in kidneys from diabetic mice. Values are means ± SE; n = 6/group. Groups are defined as in Fig. 2. *P < 0.05 compared with control group. #P < 0.05 compared with STZ-treated WT mice.

Fig. 9. Renal mRNA expression of inflammatory cytokines TNF-α (A), IL-1 (B), and IL-6 (C) in 4 groups. WldS prevented sharp elevation of these 3 inflammatory cytokines in diabetic kidneys. Values are means ± SE; n = 4/group. Groups are defined as in Fig. 2. *P < 0.05 compared with control group. #P < 0.05 compared with STZ-treated WT mice.
protein expressed in most cells, including renal cells. Thus it would have advantages in both prevention and treatment of diabetic renal injury compared with the edible reagents mentioned above.

The mechanism underlying WldS delaying the progression of diabetic renal injury may act through suppression of p38 and ERK signaling. MAPK is activated in glomeruli isolated from STZ-induced diabetic rats as well as in mesangial cells cultured under high-glucose conditions by multiple mechanisms (5, 7, 25, 48). During the early phase of DN, expression levels of p-ERK and p-p38 in both glomeruli and interstitium are higher than controls, which results in hypertrophy and extracellular matrix accumulation (15, 22, 28). In particular, the number of p-ERK-positive cells in glomeruli correlates well with the extent of glomerular lesions, whereas the number of p-p38-positive cells in the interstitium correlates with the severity of tubulointerstitial lesions (1, 37). A pharmacological ERK inhibitor and p38 inhibitor are shown as renoprotective in vitro (15, 22, 25, 45). Moreover, MKK3 gene-deficient diabetic db/db mice have a reduction in albuminuria with concomitant podocyte preservation and reduced mesangial cell activation. Meanwhile, glomerular sclerosis and tubulointerstitial injury are also attenuated (33). These evidences support our notion that the ability of WldS to inhibit MAPK phosphorylation may also contribute to its beneficial effect on diabetic renal injury.

In conclusion, we showed for the first time that WldS improved metabolic disorders and morphology changes in early experimental diabetic renal injury via slowing the reduction of the NAD+/NADH ratio, suppressing enhanced NADPH oxidase activity, inhibiting the upregulation of inflammatory cytokines, and the activation of p38 and ERK signaling.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.Z., Y.Y., X.L., Q.Y., H.B., and Q.C. provided conception and design of research; S.Z., Y.Y., J.H., L.Q., and Y.J. performed experiments; S.Z. and Y.Y. analyzed data; S.Z., Y.Y., J.H., L.Q., and Q.C. interpreted results of experiments; S.Z. prepared figures; S.Z. drafted manuscript; S.Z. and Q.C. edited and revised manuscript; S.Z., Y.Y., J.H., L.Q., Y.J., X.L., Q.Y., H.B., and Q.C. approved final version of manuscript.

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