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Role of the macula densa sodium glucose cotransporter type 1-nitric oxide synthase 1-tubuloglomerular feedback pathway in diabetic hyperfiltration

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Abstract

An increase of glomerular filtration rate (GFR) is a common observation in early diabetes and is considered a key risk factor for subsequent kidney injury. However, the mechanisms underlying diabetic hyperfiltration have not been fully clarified. Here, we tested the hypothesis that macula densa neuronal nitric oxide synthase (NOS1) is upregulated via sodium glucose cotransporter type 1 (SGLT1) in diabetes, which then inhibits tubuloglomerular feedback (TGF) promoting glomerular hyperfiltration. Therefore, we examined changes in cortical NOS1 expression and phosphorylation, nitric oxide production in the macula densa, TGF response, and GFR during the early stage of insulin-deficient (Akita) diabetes in wild-type and macula densa-specific NOS1

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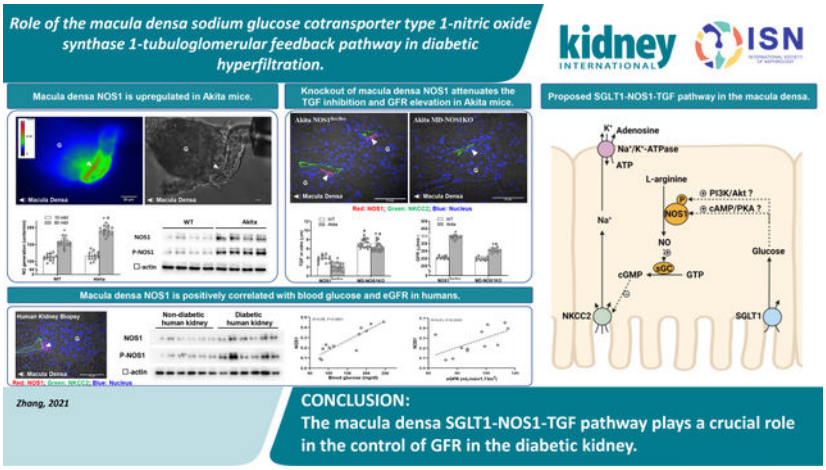
knockout mice. A set of sophisticated techniques including microperfusion of juxtaglomerular apparatus *in vitro*, micropuncture of kidney tubules *in vivo*, and clearance kinetics of plasma fluorescent-sinistrin were employed. Complementary studies tested the role of SGLT1 in SGLT1 knockout mice and explored NOS1 expression and phosphorylation in kidney biopsies of cadaveric donors. Diabetic mice had upregulated macula densa NOS1, inhibited TGF and elevated GFR. Macula densa-selective NOS1 knockout attenuated the diabetes-induced TGF inhibition and GFR elevation. Additionally, deletion of SGLT1 prevented the upregulation of macula densa NOS1 and attenuated inhibition of TGF in diabetic mice. Furthermore, the expression and phosphorylation levels of NOS1 were increased in cadaveric kidneys of diabetics and positively correlated with blood glucose as well as estimated GFR in the donors. Thus, our findings demonstrate that the macula densa SGLT1-NOS1-TGF pathway plays a crucial role in the control of GFR in diabetes.

Graphical Abstract



Keywords

diabetes; renal hemodynamic; glomerular filtration rate; tubuloglomerular feedback; neuronal nitric oxide synthase; sodium glucose cotransporter type 1

INTRODUCTION

Elevated glomerular filtration rate (GFR), or glomerular hyperfiltration, is a common observation in the early stage of diabetes. It occurs in over 70% of type 1[1, 2] and approximately 50% of type 2[2–5] diabetic patients, and is associated with a higher prevalence of diabetic nephropathy and worse prognosis.[1, 2, 4, 6] Nevertheless, mechanisms underlying the development of glomerular hyperfiltration in diabetes have not been fully clarified.

We have recently identified a new mechanism for the acute hyperglycemia–induced glomerular hyperfiltration, wherein an increase of luminal glucose load at the macula densa enhances neuronal nitric oxide synthase (NOS1)-derived nitric oxide (NO) generation through sodium-glucose cotransporter 1 (SGLT1), thus blunting the tubuloglomerular

feedback (TGF) response and consequently increasing GFR (macula densa SGLT1-NOS1-TGF pathway).[7–9] However, the significance of this macula densa SGLT1-NOS1-TGF pathway in the long-term control of GFR in diabetes remains to be determined.

In the present study, we hypothesized that in early diabetes, increases of glucose load at the macula densa upregulate the expression and activity of macula densa NOS1 via SGLT1, which inhibits the TGF responsiveness and thereby promotes the development of glomerular hyperfiltration. A set of sophisticated techniques including microperfusion of juxtaglomerular apparatus (JGA) *in vitro*, micropuncture of renal tubules *in vivo*, and clearance kinetics of plasma FITC-sinistrin in conscious animals were employed to examine the changes of NOS1 expression and phosphorylation as well as NO production in the macula densa, TGF response, and GFR during the early stage of insulin-deficient (Akita) diabetes in wild-type (WT), macula densa–specific NOS1 knockout (MD-NOS1KO), and SGLT1 knockout (SGLT1KO) mice. Furthermore, the clinical significance of this pathway in diabetic hyperfiltration was evaluated by correlating macula densa NOS1 expression and phosphorylation with blood glucose as well as estimated GFR (eGFR) in non-diabetic and diabetic cadaveric donors.

METHODS

All animal experiments were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals with all animal protocols approved by the local Institutional Animal Care and Use Committee. As DBA/2J genetic background enhances the diabetic phenotypes including hyperglycemia as well as glomerular hyperfiltration in Akita mice,[10–12] the Akita mice on DBA/2J genetic background (#007562, Jackson Laboratories, Bar Harbor, ME) were used as diabetic models, and the DBA/2J mice (#000671, Jackson Laboratories, Bar Harbor, ME) were used as non-diabetic controls. The MD-NOS1KO ($NKCC2^{cre}/NOS1^{flox/flox}$) mice on C57BL/6J genetic background have been previously generated by crossing $NKCC2^{cre}$ mice with $NOS1^{flox/flox}$ mice.[13] In the present study, MD-NOS1KO mice were first backcrossed to the DBA/2J mice for 4 generations and then crossed to the Akita mice to generate Akita MD-NOS1KO mice. Littermate Akita $NOS1^{flox/flox}$ mice were used as diabetic controls, and littermate MD-NOS1KO and $NOS1^{flox/flox}$ mice were used as non-diabetic controls. All these mice were housed individually at 23°C on a 12:12-hour light-dark cycle, with food and water ad libitum (#2918; Envigo, Indianapolis, IN). Additionally, the generation, husbandry, and basic phenotype of SGLT1KO as well as Akita SGLT1KO mice on DBA/2J genetic background have been previously described.[10] Detailed methods are provided in the Supplementary Materials.

RESULTS

Part I. The Development of Glomerular Hyperfiltration in Akita Mice is Associated with Upregulated Macula Densa NOS1 and Inhibited TGF.

Blood glucose concentration was significantly higher in Akita mice (e.g., 470±27 mg/dl at 12 weeks of age) than in WT mice (145±17 mg/dl at 12 weeks of age) (Figure 1A). GFR

was ~50% higher in Akita mice (e.g., 393±13 μl/min at 12 weeks of age) than in WT mice (259±11 μl/min at 12 weeks of age) (Figure 1B).

To determine the changes in TGF responsiveness during the early stage of diabetes, we measured the TGF response *in vivo* with micropuncture in Akita and WT mice at 12 weeks of age. In WT mice, when tubular perfusion rate of artificial tubular fluid (ATF) was increased from 0 to 40 nl/min, stop-flow pressure (P_{sf}) fell from 37.6±2.1 to 32.7±2.6 mmHg, and thereby the TGF response *in vivo*, as indicated by the change in the P_{sf} (ΔP_{sf}), was 4.9±0.8 mmHg (Figure 1C). In Akita mice, the TGF response *in vivo*, as indicated by ΔP_{sf}, was 2.0±0.7 mmHg (Figure 1D), which was ~60 % less compared with WT mice (Figure 1E).

To eliminate systemic confounding factors such as hormones and sympathetic activity, we also measured the TGF response *in vitro* in isolated and double perfused JGAs. In WT mice, when NaCl concentration in tubular perfusate was increased from 10 to 80 mM, the diameter of afferent arteriole (Af-Art) decreased from 15.8±1.0 to 11.9±0.8 μm, and thereby the TGF response *in vitro*, as indicated by the change in Af-Art diameter, was 3.9±0.6 μm (Figure 1F). In Akita mice, the TGF response *in vitro*, as indicated by the change in Af-Art diameter, was 2.1±0.5 μm (Figure 1G), which was ~45 % less compared with WT mice (Figure 1H).

To determine the changes in macula densa NO production during the early stage of diabetes, we measured the TGF-induced NO generation in the macula densa of isolated and perfused JGAs with the fluorescent NO probe 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-2 DA) in Akita and WT mice at 12 weeks of age (Figure 1I). In WT mice, when NaCl concentration in tubular perfusate was switched from 10 to 80 mM, macula densa NO production increased by 38.0±4.7 % from 112.5±9.8 to 155.1±12.5 units/min. In Akita mice, when NaCl concentration in tubular perfusate was switched from 10 to 80 mM, macula densa NO generation increased by 64.5±10.4 % from 116.8±11.9 to 191.3±12.8 units/min, which was significantly greater than in WT mice (Figure 1J).

To determine the changes in macula densa NOS1 expression and phosphorylation during the early stage of diabetes, we measured the protein abundances of NOS1 and NOS1 phosphorylated at Ser1417 (P-NOS1) in the renal cortex, where most of the NOS1 comes from the macula densa,[7, 14, 15] in Akita and WT mice at 12 weeks of age (Figure 1K). NOS1 levels (Figure 1L) as well as P-NOS1/ NOS1 ratios (Figure 1M) were 1.81±0.25-fold and 1.49±0.23-fold higher, respectively, in Akita mice compared with WT mice.

These results demonstrate that the development of glomerular hyperfiltration in the early stage of insulin-deficient diabetes is associated with upregulation of macula densa NOS1 as well as inhibition of TGF.

Part II. Selective Deletion of Macula Densa NOS1 Attenuates Diabetes-Induced Inhibition of TGF and Elevation of GFR in Akita Mice.

Blood glucose concentration was not significantly different in Akita MD-NOS1KO mice compared with Akita $NOS1^{flox/flox}$ mice, which indicates that the insulin deficiency (Akita)-induced diabetic condition was not affected by the genetic recombination with the MD-

NOS1KO mouse line (Figure 2A). Double-immunofluorescence staining of NOS1 and NKCC2 in the kidney slices of Akita MD-NOS1KO mice confirmed the absence of NOS1 expression in the macula densa, which indicates that the deletion of macula densa NOS1 was not affected by the genetic recombination with the Akita mouse line (Figure 2B).

To determine the significance of macula densa NOS1 in the inhibition of TGF during the early stage of diabetes, we measured the TGF response *in vivo* with micropuncture in Akita and non-diabetic MD-NOS1KO as well as $NOS1^{flox/flox}$ mice at 12 weeks of age. In $NOS1^{flox/flox}$ mice, the TGF response *in vivo*, as indicated by ΔP_{sf}, was ~60% less in the diabetic group (1.9±1.1 mmHg) compared with the non-diabetic group (5.1±0.8 mmHg) (Figures 2C, 2D and 2G). In MD-NOS1KO mice, the TGF response *in vivo* (which was already enhanced) was only ~21 % less in the diabetic group (6.4±1.2 mmHg) compared with the non-diabetic group (8.1±0.9 mmHg) (Figures 2E, 2F and 2G). The diabetes-induced inhibition of TGF *in vivo* was significantly less in the MD-NOS1KO mice than the $NOS1^{flox/flox}$ mice (Figure 2G).

To eliminate systemic confounding factors, we also measured the TGF response *in vitro* in isolated and double perfused JGAs. In $NOS1^{flox/flox}$ mice, the TGF response *in vitro*, as indicated by the change of Af-Art diameter, was ~42 % less in the diabetic group (2.2±0.6 μm) compared with the non-diabetic group (3.8±0.7 μm) (Figures 2H, 2I and 2L). In the MD-NOS1KO mice, the TGF response *in vitro* (which was already enhanced) was only ~12 % less in the diabetic group (6.2±1.0 μm) compared with the non-diabetic group (7.1±0.9 μm) (Figures 2J, 2K and 2L). The diabetes-induced inhibition of TGF *in vitro* was significantly less in the MD-NOS1KO mice than the $NOS1^{flox/flox}$ mice (Figure 2L).

To determine the significance of macula densa NOS1 in the development of glomerular hyperfiltration during the early stage of diabetes, we measured GFR by assessing the clearance kinetics of plasma FITC-sinistrin in Akita and non-diabetic MD-NOS1KO as well as $NOS1^{flox/flox}$ mice once a week from 8 weeks to 12 weeks of age. In $NOS1^{flox/flox}$ mice, GFR was ~52% higher in the diabetic group (e.g., 393±13 μl/min at 12 weeks of age) than in the non-diabetic group (258±10 μl/min at 12 weeks of age). In MD-NOS1KO mice, GFR was only ~23 % higher in the diabetic group (e.g., 306±14 μl/min at 12 weeks of age) than in the non-diabetic group (249±12 μl/min at 12 weeks of age). The diabetes-induced rise of GFR was significantly less in the MD-NOS1KO mice than the $NOS1^{flox/flox}$ mice (Figure 2M).

These results demonstrate that selective deletion of NOS1 from the macula densa reduces the inhibition of TGF as well as the elevation of GFR in the early stage of insulin-deficient diabetes.

Part III. Deletion of SGLT1 Attenuates Diabetes-Induced Upregulation of Macula Densa NOS1 and Inhibition of TGF in Akita Mice.

To determine the significance of SGLT1 in the upregulation of macula densa NOS1 during the early stage of diabetes, we measured the protein abundances of NOS1 and P-NOS1 in the renal cortex of littermate non-diabetic SGLT1KO and Akita SGLT1KO mice at 17 weeks of age (Figure 3A). Neither NOS1 levels (Figure 3B) nor P-NOS1/ NOS1 ratios (Figure 3C)

were significantly different in Akita SGLT1KO mice compared with non-diabetic SGLT1KO mice. These results indicate that deletion of SGLT1 inhibits the upregulation of macula densa NOS1 in the early stage of insulin-deficient diabetes.

To determine the significance of SGLT1 in the inhibition of TGF during the early stage of diabetes, we measured the TGF response in vitro in isolated and double perfused JGAs of littermate non-diabetic SGLT1KO and Akita SGLT1KO mice at 16 weeks of age. In SGLT1KO mice, the TGF response *in vitro*, as indicated by the change of Af-Art diameter, was 4.1±0.5 μm (Figure 3D and 3F). In Akita SGLT1KO mice, the TGF response *in vitro*, as indicated by the change of Af-Art diameter, was 3.1±0.5 μm (Figure 3E and 3F). The diabetes-induced inhibition of TGF in vitro was ~25% in the SGLT1KO mice (Figure 3F), which was significantly less than that in the WT mice (~45%) (Figure 1H). These results indicate that deletion of SGLT1 reduces the upregulation of macula densa NOS1 as well as the inhibition of TGF in the early stage of insulin-deficient diabetes.

Part IV. The Development of Glomerular Hyperfiltration in Diabetic Patients is Associated with Upregulated Macula Densa NOS1.

To identify the localization of NOS1 in human kidney, we performed double immunofluorescence staining of NOS1 and NKCC2 in renal biopsy sections. As shown in the images (Figure 4A), NOS1 is primarily expressed at the macula densa (co-localized with NKCC2) within the renal cortex of human kidney, along with minor distribution at other segments (e.g., collecting duct).

To determine the changes in macula densa NOS1 expression and phosphorylation in diabetic patients, we measured the protein abundances of NOS1 and P-NOS1 in the renal biopsy tissues from cadaveric donors with and without diabetes. NOS1 levels (Figure 4B and 4C) and P-NOS1/NOS1 ratios (Figure 4B and 4D) were 2.12±0.59-fold and 1.91±0.63-fold higher, respectively, in diabetic donors compared with non-diabetic donors. Moreover, both NOS1 and P-NOS1/NOS1 levels in these cadaveric kidneys were positively correlated with blood glucose concentration (Figure 4E, r=0.89 and Figure 4E, r=0.72) as well as eGFR (Figure 4G, r=0.61 and Figure 4H, r=0.58) measured in the donors.

These results indicate that macula densa NOS1 is upregulated in diabetic patients and associated with development of glomerular hyperfiltration.

DISCUSSION

The present study demonstrated the significance of the macula densa SGLT1-NOS1-TGF pathway in the control of GFR during the early stage of diabetes by showing that (1) the development of glomerular hyperfiltration in Akita mice was associated with inhibited TGF responsiveness as well as upregulated expression, phosphorylation and activity of macula densa NOS1; (2) selective deletion of macula densa NOS1 attenuated the diabetes-induced TGF inhibition and GFR elevation in Akita mice; (3) deletion of SGLT1 prevented the upregulation of NOS1 and reduced the inhibition of TGF in Akita mice; (4) the expression and phosphorylation levels of NOS1 were increased in diabetic cadaveric kidneys and positively correlated with blood glucose as well as eGFR in the donors.

Several mechanisms have been implicated in diabetic glomerular hyperfiltration, primarily including vascular and tubular theories. According to the vascular hypothesis, glomerular hyperfiltration results from the imbalance between vasoconstrictive and vasodilatory factors in diabetes.[1, 2, 16] The tubular thesis proposes that the tubular growth as well as the upregulation of sodium-glucose cotransporter 2 (SGLT2) in diabetes enhances proximal tubular reabsorption, which reduces the NaCl delivery to the macula densa and thereby increases GFR via TGF (SGLT2-NaCl-TGF pathway).[17–20] Recently, we identified a new mechanism for the acute hyperglycemia–induced glomerular hyperfiltration, the macula densa SGLT1-NOS1-TGF pathway.[7–9] However, the long-term significance of this mechanism in the pathogenesis of glomerular hyperfiltration in diabetes had not been determined, which was examined in the present study using an insulin-deficient (Akita) diabetic mouse model.

The macula densa is a group of specialized epithelial cells located at the distal end of TAL, serving as a sensor of tubular fluid. The TGF describes an important intrinsic mechanism in the control of renal hemodynamics wherein an increase of NaCl delivery to the macula densa promotes the release and formation of ATP and/or adenosine, which constricts the Af-Art and induces an inhibition of single-nephron GFR.[21–24] The effect of diabetes on TGF has been extensively investigated in various rodent models. The TGF response, as indicated by ΔP_{sf}, proximal-distal differences of single nephron GFR, or homeostatic efficiency of TGF was found to be attenuated or reset in streptozotocin-induced diabetic rats,[17–19, 25] db/db mice[26, 27] as well as Akita mice.[28] Consistent with these previous findings, we found that both the TGF response measured *in vitro* by microperfusion and *in vivo* by micropuncture were weakened in Akita mice compared with WT mice.

Although the mechanisms underlying the diabetes-induced modulation of TGF responsiveness have not been elucidated, multiple studies have indicated a potential role of the macula densa NOS1. NOS1 is the predominant NOS isoform expressed in the macula densa[14, 15] and the NO derived from macula densa NOS1 buffers or attenuates the TGF vasoconstrictor tone via a cGMP-dependent signaling pathway.[13, 15, 29–31] It has been demonstrated that the renal hemodynamic changes during the early stage of diabetes are associated with increased NO production.[32–35] Moreover, selective NOS1 inhibition induces a greater reduction in GFR in diabetic animals than non-diabetics,[27, 35–37] and GFR is not further decreased by non-selective NOS inhibitors in the presence of a NOS1 inhibitor.[36] Consistent with these previous findings, we found that NOS1 expression and phosphorylation at Ser1417 (which activates NOS1 via cAMP-dependent protein kinase)[38–40] in the renal cortex, where most of the NOS1 comes from the macula densa,[7, 14, 15] were significantly increased in Akita mice compared with WT mice. In accordance with the changes in expression and phosphorylation, the activity of NOS1 in the macula densa, as indicated by the TGF-induced NO production, was also found to be markedly enhanced in Akita mice compared with WT mice. These results demonstrate that the development of glomerular hyperfiltration in the early stage of diabetes is associated with upregulation of macula densa NOS1 as well as inhibition of TGF responsiveness. Nevertheless, whether the inhibited TGF and the upregulated macula densa NOS1 are causal factors or consequences of glomerular hyperfiltration remains elusive.

Recently, our laboratory generated a MD-NOS1KO mouse model (NKCC2^{cre}/NOS1$^{flox/flox}$) by crossing a NKCC2^{cre} line with a NOS1$^{flox/flox}$ line.[13] As the NOS1$^{flox/flox}$ mouse line[41] (kindly provided by Dr. Paul Huang) targets exon 6, a common exon for all the splice variants of NOS1, the excision by NKCC2 promoter-driven Cre recombinase results in complete ablation of all the splice variants of NOS1 in the macula densa. In the non-diabetic control condition, MD-NOS1KO mice have augmented TGF responses, but their kidney functions are normal without significant renal injury.[13] Thus, in the present study, we used this MD-NOS1KO mouse model to examine the significance of macula densa NOS1 in the regulation of TGF and GFR during the early stage of diabetes. Diabetic MD-NOS1KO mice were generated by crossing MD-NOS1KO mice with Akita mice, and further characterization showed that neither the blood glucose level nor the NOS1 expression in the macula densa was affected by this genetic recombination. We found that the macula densa-specific NOS1 deletion significantly attenuated the diabetes-induced TGF inhibition and GFR elevation, which indicated that macula densa NOS1 plays an essential role in the modulation of TGF as well as the development of glomerular hyperfiltration in Akita mice, a murine model of insulin-deficient diabetes.

Moreover, the clinical significance of macula densa NOS1 in diabetic hyperfiltration was evaluated by correlating macula densa NOS1 with blood glucose and GFR in cadaveric kidney donors. We found that NOS1 was primarily expressed at the macula densa (co-localized with NKCC2) within the human kidney biopsy tissue (renal cortex); both the expression and phosphorylation levels of NOS1 were significantly higher in diabetic kidneys than non-diabetic kidneys, and positively correlated with blood glucose concentration as well as eGFR in these donors. These results indicated that the findings in mice on the mechanisms for diabetic hyperfiltration might be extrapolated into humans, such that macula densa NOS1-driven attenuation of TGF response may also play a pivotal role in the development of glomerular hyperfiltration in diabetic patients.

SGLT1, a low-capacity and high-affinity Na^+/glucose transmembrane transporter, is involved in the regulation of macula densa NOS1 in diabetes. Besides its localization in the proximal tubule, SGLT1 has also been found to be expressed on the apical membrane of the TAL as well as the macula densa in both rodent[42, 43] and human[7] kidneys. Furthermore, analysis of kidney single-cell RNA-sequencing data reveals that SGLT1 has the highest transcriptional expression level among all known glucose transporters in the macula densa cells.[7] We previously showed by immunostaining that gene knockout of SGLT1 prevented the upregulation of macula densa NOS1 expression in Akita mice, and the absence of SGLT1 also attenuated the associated glomerular hyperfiltration.[10] Here we used the same samples to confirm and extend these findings by Western blotting, showing that the deletion of SGLT1 not only prevented the increase of renal cortical NOS1 expression but also its phosphorylation in Akita mice, which further suggests that the upregulation of macula densa NOS1 in insulin-deficient diabetes is mediated via SGLT1.

Moreover, there are insights from non-macula densa cells on how glucose can affect NOS1 expression and activity. Glucose can activate the cyclic adenosine monophosphate (cAMP) / protein kinase A (PKA) signaling pathway,[44–46] which increases NOS1 expression by upregulating the transcription factor cAMP response element-binding protein (CREB).[47, 48]

Glucose can also stimulate the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway,[49, 50] which enhances the transcription of NOS1 gene via the CREB transcription factor.[51–53] Additionally, both the PI3K/Akt[54, 55] and cAMP/PKA[40, 48, 56] pathways play important roles in the regulation of the phosphorylation of NOS1. Therefore, we speculate that in diabetes, the glucose load transported from tubular fluid into the macula densa cells via SGLT1 activates the cAMP /PKA and PI3K/Akt signaling pathways, which increases the expression of NOS1 via CREB and upregulates the activity of NOS1 by phosphorylation at Ser1417 (Figure 5).

While the macula densa SGLT1-NOS1-TGF pathway is the focus of the present study, we are aware that the ablation of macula densa NOS1 or SGLT1 does not completely block the rise of GFR in diabetic mice, and other mechanisms, particularly the SGLT2-NaCl-TGF pathway, also contribute. We propose that the SGLT1-NOS1-TGF and SGLT2-NaCl-TGF pathways are separate mechanisms operating in parallel that additively promote the development of glomerular hyperfiltration in the early diabetic kidney, consistent with our recent studies.[10] We assume that hyperglycemia increases glucose filtration through the glomerulus and thereby enhances the SGLT2-mediated reabsorption in the proximal tubule, which lowers the NaCl delivery to the macula densa and reduces the vasoconstrictor TGF tone;[18–20] meanwhile, the increased luminal glucose load at the macula densa is sensed by SGLT1, where it upregulates NOS1 and further attenuates the vasoconstrictor tone of TGF.

In conclusion, the present study demonstrates that the macula densa SGLT1-NOS1-TGF pathway plays a critical role in the control of GFR during the early stage of a murine model of insulin-deficient diabetes, with complementing evidence presented in the human diabetic kidney. These findings not only establish a novel pathophysiological mechanism but also provide a potential therapeutic target for diabetic glomerular hyperfiltration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DISCLOSURE STATEMENT

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TRANSLATIONAL STATEMENT

Glomerular hyperfiltration is a common observation in the early stage of diabetes, which is associated with increased risk for subsequent kidney injury and worse prognosis. The present study demonstrates that the macula densa sodium glucose cotransporter type 1 - neuronal nitric oxide synthase - tubuloglomerular feedback pathway plays a critical role in the control of glomerular filtration rate in a murine model of insulin deficient diabetes, with complementing evidence presented in the human diabetic kidney. These findings not only establish a novel pathophysiological mechanism but also provide a potential therapeutic target for diabetic glomerular hyperfiltration and even kidney injury.

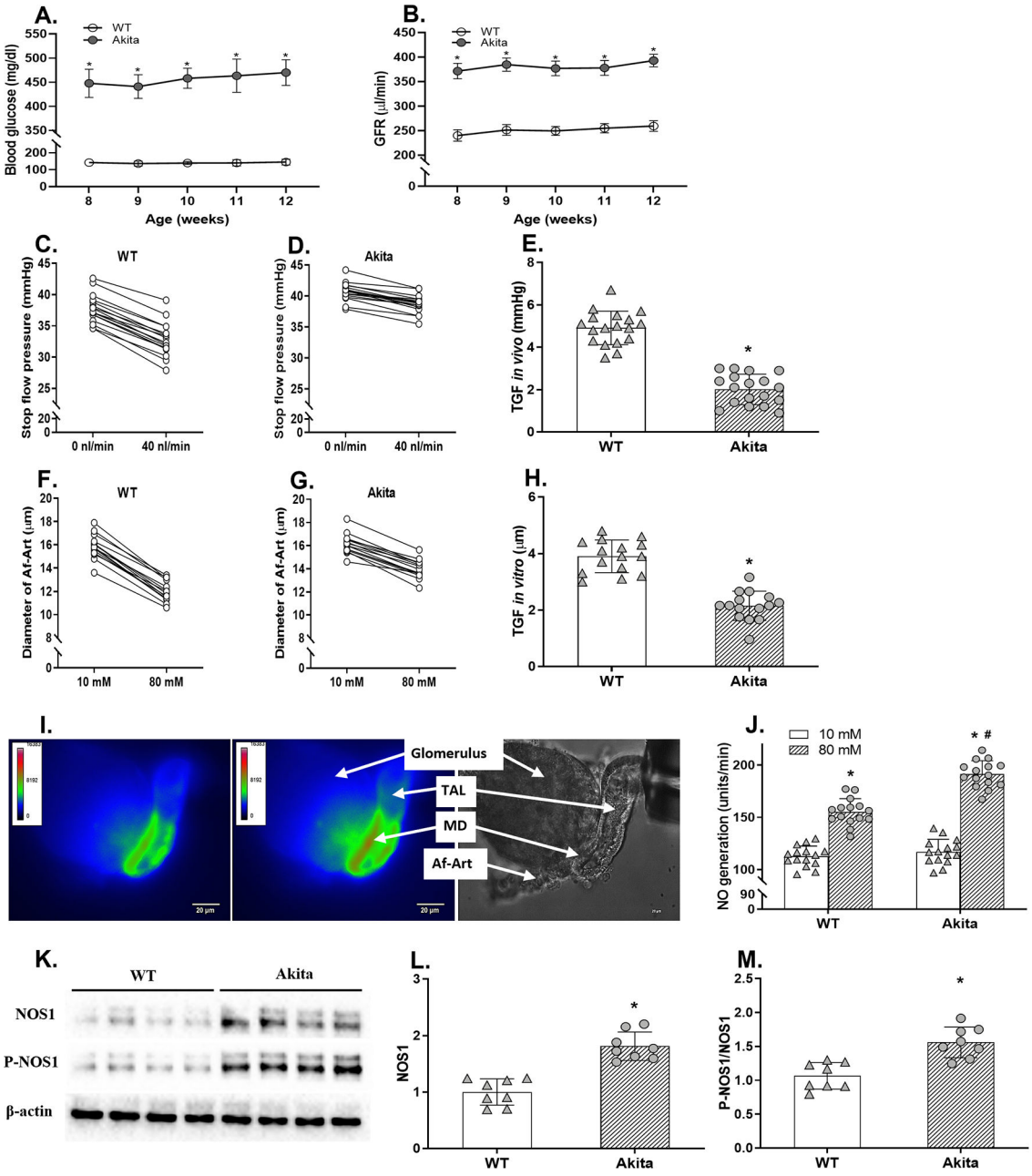

Figure 1. Blood glucose, GFR, TGF response as well as macula densa NOS1 expression and activity in Akita mice.

(A) The blood glucose concentration in Akita and WT mice. n=10–12 per group; *P<0.01 vs WT. **(B)** The GFR in Akita and WT mice. n=10 per group; *P<0.01 vs WT. **(C-E)** The TGF response *in vivo* in Akita and WT mice. n=18 tubules/6 mice per group; *P<0.01 vs WT. **(F-H)** The TGF response *in vitro* in Akita and WT mice. n=15 nephrons per group; *P<0.01 vs WT. **(I)** The measurement of TGF-induced NO generation by the macula densa in isolated and perfused JGA with DAF-2 DA. The bright field image exhibits the anatomic structure of the isolated and perfused JGA. The florescent image shows the NO production in the DAF-2 DA-loaded JGA. **(J)** The TGF–induced NO generation at the macula densa in

Akita and WT mice. n=15 nephrons/group; *P<0.01 vs 10 mM, #P<0.01 vs WT. **(K)** The immunoblots of renal cortical NOS1, P-NOS1 and loading control of β-actin in Akita and WT mice. **(L-M)** The renal cortical NOS1 and P-NOS1/NOS1 levels in Akita and WT mice. n=8 per group; *P<0.01 vs WT. Statistical difference in (A and B) was calculated by two-way repeated measures ANOVA followed by Sidak multiple comparisons test. Statistical difference in (C-H, L-M) was calculated by t-test. Statistical difference in (J) was calculated by two-way ANOVA followed by Sidak multiple comparisons test.

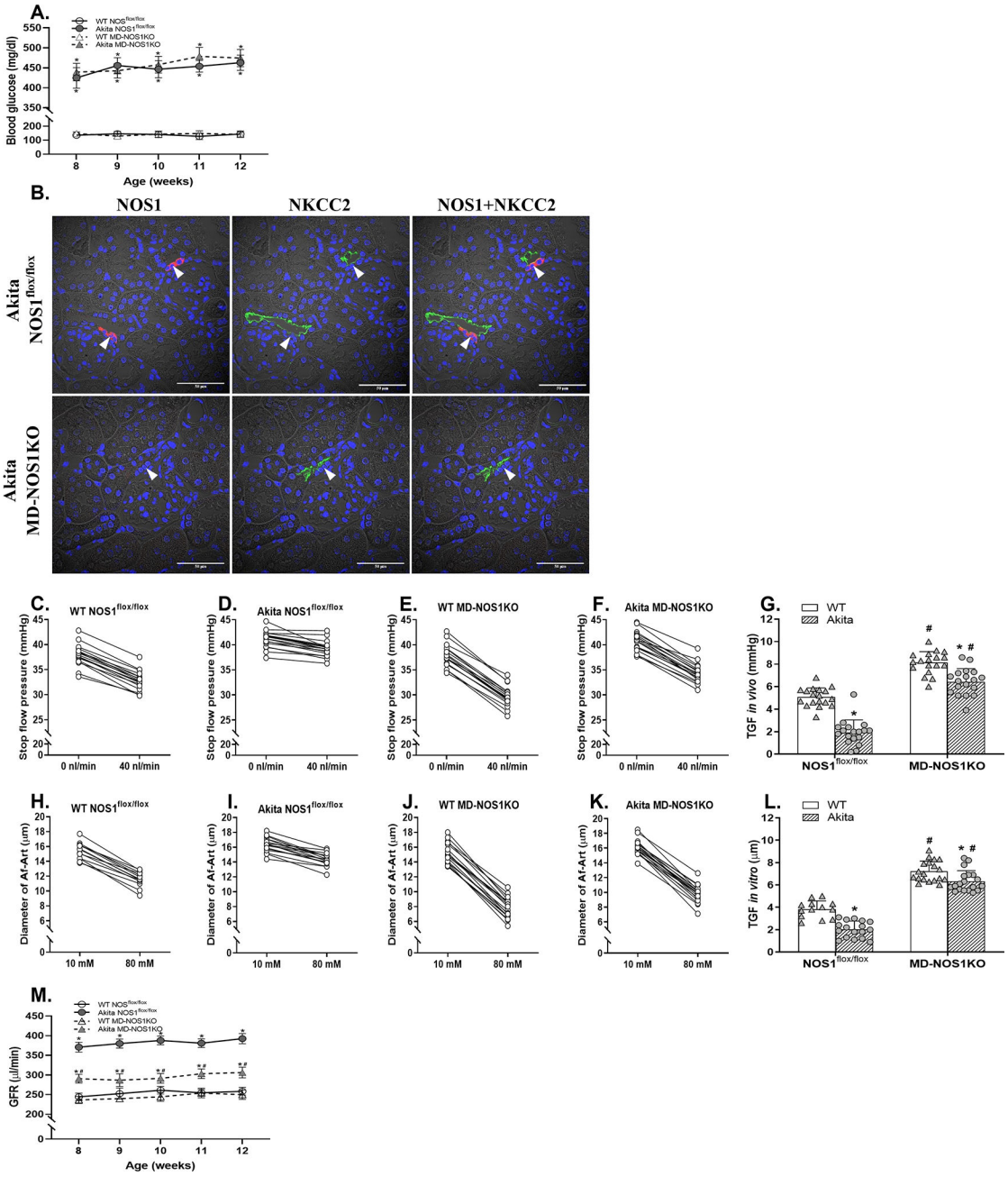

Figure 2. Effect of macula densa-selective NOS1 deletion on blood glucose, TGF responsiveness and GFR in Akita mice.

(A) Blood glucose concentration in WT NOS1$^{flox/flox}$, Akita NOS1$^{flox/flox}$, WT MD-NOS1KO and Akita MD-NOS1KO mice. **(B)** Double immunofluorescence staining of NOS1 and NKCC2 in kidney slices of Akita NOS1$^{flox/flox}$ and MD-NOS1KO mice. Red: NOS1; Green: NKCC2; Blue: nucleus; Arrow: macula densa. **(C-G)** TGF response *in vivo* in WT NOS1$^{flox/flox}$, Akita NOS1$^{flox/flox}$, WT MD-NOS1KO and Akita MD-NOS1KO mice. n=18 tubules/6 mice per group; *P<0.01 vs WT; $^{\#}$P<0.01 vs NOS1$^{flox/flox}$. **(H-L)** TGF response *in vitro* in WT NOS1$^{flox/flox}$, Akita NOS1$^{flox/flox}$, WT MD-NOS1KO and Akita MD-NOS1KO mice. n=13–15 nephrons per group; *P<0.05 vs WT; $^{\#}$P<0.01 vs

NOS1$^{flox/flox}$. **(M)** GFR in WT NOS1$^{flox/flox}$, Akita NOS1$^{flox/flox}$, WT MD-NOS1KO and Akita MD-NOS1KO mice. n=10 per group; *P<0.01 vs WT; $^{\#}$P<0.01 vs NOS1$^{flox/flox}$. Statistical difference in (A and M) was calculated by two-way repeated measures ANOVA followed by Sidak multiple comparisons test. Statistical difference in (C-F, H-K) was calculated by t-test. Statistical difference in (G and L) was calculated by two-way ANOVA followed by Sidak multiple comparisons test.

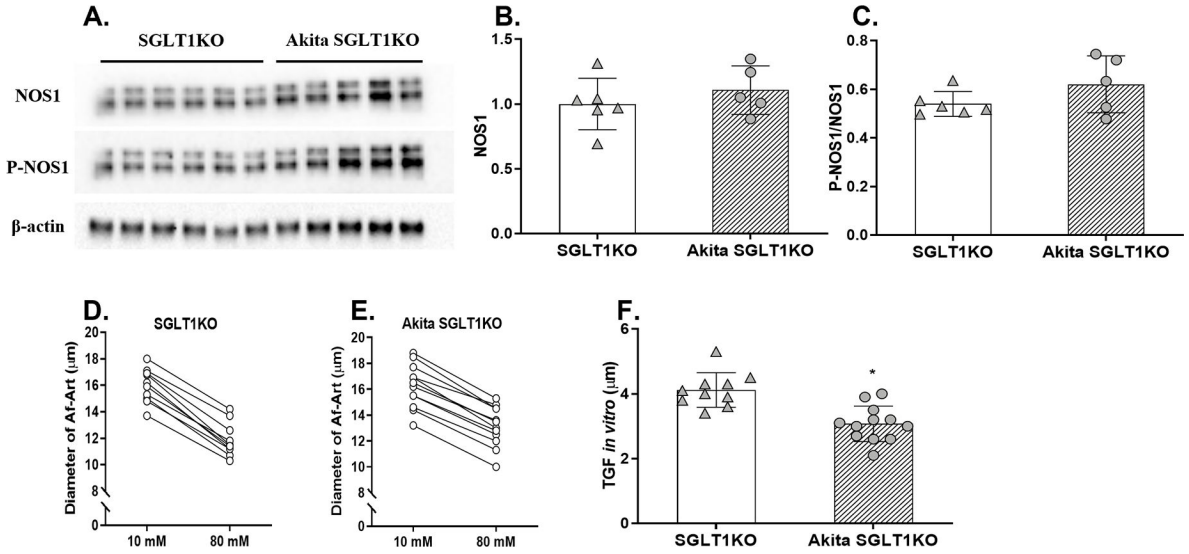

Figure 3. Effect of SGLT1 deletion on macula densa NOS1 expression and phosphorylation in Akita mice.

(A) Immunoblots of renal cortical NOS1, P-NOS1 and loading control of β-actin in SGLT1KO and Akita SGLT1 KO mice. **(B** and **C)** The renal cortical NOS1 and P-NOS1/NOS1 levels in SGLT1KO and Akita SGLT1 KO mice. n=5–6 per group. **(D-F)** TGF response *in vitro* in SGLT1KO and Akita SGLT1KO mice. n=10–12 nephrons per group; *P<0.01 vs SGLT1KO. Statistical difference in (B-C and D-F) was calculated by t-test.

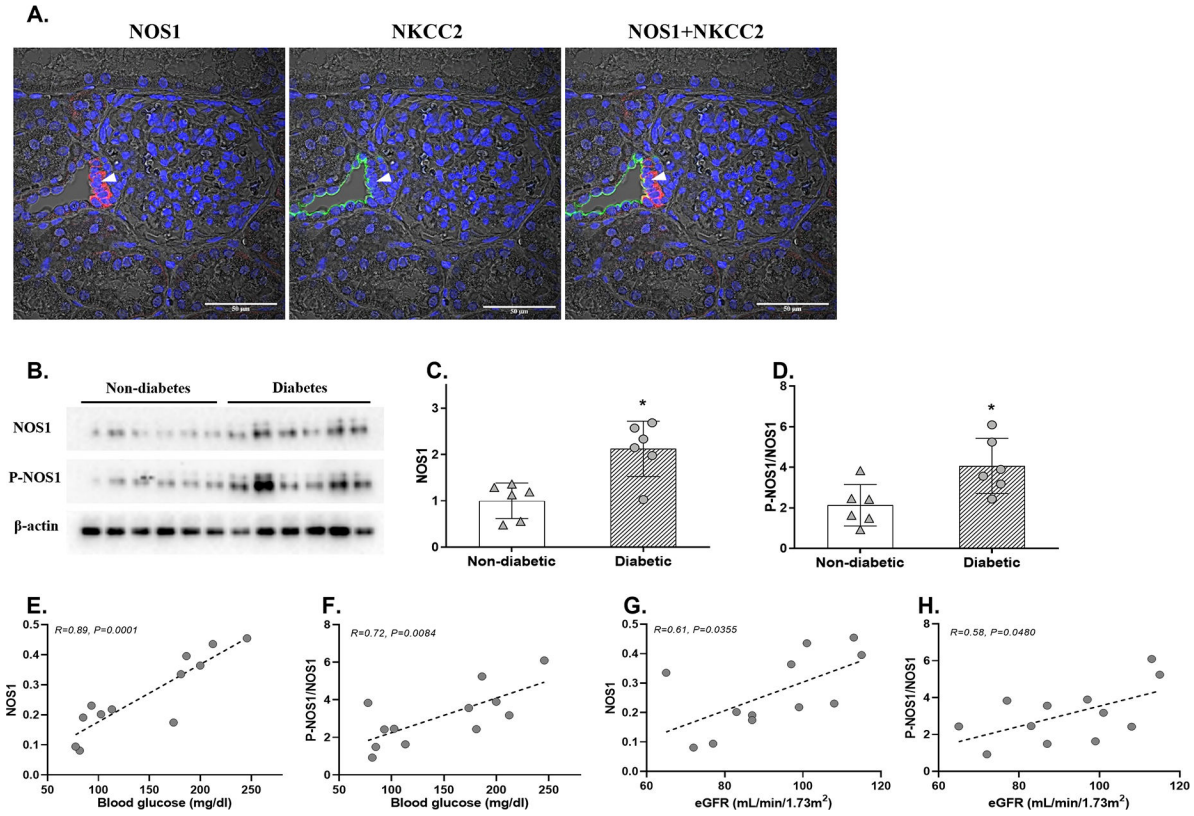

Figure 4. Correlation of macula densa NOS1 with blood glucose and eGFR in cadaveric kidney donors.

(A) Double immunofluorescence staining of NOS1 and NKCC2 in human kidney biopsy slices. Red: NOS1; Green: NKCC2; Blue: nucleus; Arrow: macula densa. **(B)** Immunoblots of NOS1, P-NOS1 and the loading control of β-actin in the kidney biopsies from diabetic and non-diabetic cadaveric donors. **(C-D)** NOS1 and P-NOS1/NOS1 levels in kidney biopsies from diabetic and non-diabetic cadaveric donors. n=6 per group; *P<0.05 vs non-diabetic. **(E-F)** Correlation of NOS1 and P-NOS1/NOS1 with blood glucose in cadaveric kidney donors. **(G-H)** Correlation of NOS1 and P-NOS1/NOS1 with eGFR in cadaveric kidney donors. Statistical difference in (C and D) was calculated by t-test. Statistical difference in (E-H) was calculated by Pearson's R.

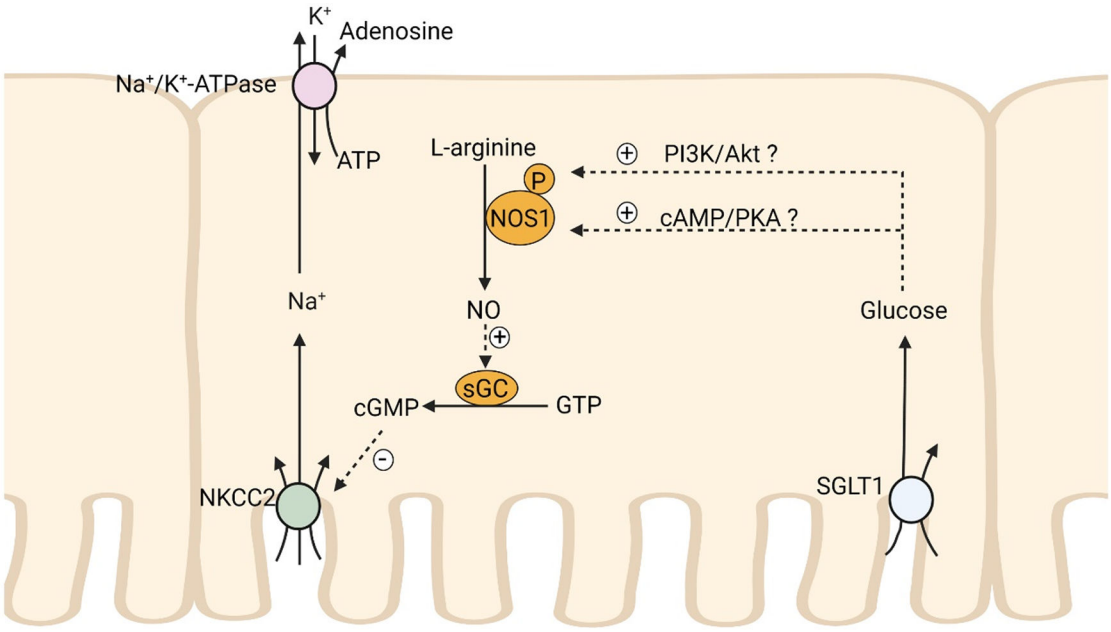

Figure 5. Proposed macula densa SGLT1-NOS1-TGF pathway in the early diabetic kidney.

In diabetic kidney, an increase of luminal glucose concentration at the macula densa is sensed by SGLT1. The glucose load transported from tubular fluid into the macula densa cells via SGLT1 could activate the cAMP /PKA and PI3K/Akt signaling pathways, which would upregulate the expression and phosphorylation levels of NOS1. NO derived from NOS1 in the macula densa inhibits TGF (mediated by NKCC2) via a cGMP-dependent mechanism.