Maintenance of Endothelial Guanosine Triphosphate Cyclohydrolase I Ameliorates Diabetic Nephropathy

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ABSTRACT

In diabetes, endothelial nitric oxide synthase (eNOS) produces superoxide anion rather than nitric oxide, referred to as "eNOS uncoupling," which may contribute to endothelial dysfunction, albuminuria, and diabetic nephropathy. Reduced levels of endothelium-derived tetrahydrobiopterin (BH4), an essential cofactor for eNOS, promote eNOS uncoupling. Accelerated degradation of guanosine triphosphate cyclohydrolase I (GTPCH I), the rate-limiting enzyme in BH4 biosynthesis, also occurs in diabetes, suggesting that GTPCH I may have a role in diabetic microvascular disease. Here, we crossed endothelium-dominant GTPCH I transgenic mice with Ins2+/Akita diabetic mice and found that endothelial overexpression of GTPCH I led to higher levels of intrarenal BH4 and lower levels of urinary albumin and reactive oxygen species compared with diabetic control mice. Furthermore, GTPCH I overexpression attenuated the hyperpermeability of macromolecules observed in diabetic control mice. In addition, we treated Ins2+/Akita mice with metformin, which activates AMP-activated protein kinase (AMPK) and thereby slows the degradation of GTPCH I; despite blood glucose levels that were similar to untreated mice, those treated with metformin had significantly less albuminuria. Similarly, in vitro, treating human glomerular endothelial cells with AMPK activators attenuated glucose-induced reductions in phospho-AMPK, GTPCH I, and coupled eNOS. Taken together, these data suggest that maintenance of endothelial GTPCH I expression and the resulting improvement in BH4 biosynthesis ameliorate diabetic nephropathy.

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reported to play an important role in diabetic endothelial dysfunction.12,13 Thus, the prevention or reversal of eNOS uncoupling may aid in the maintenance of endothelial function.

One of the mechanisms involved in eNOS uncoupling is reduced levels of tetrahydrobiopterin (BH4), an essential cofactor for eNOS.14,15 We previously reported that the imbalance between NO and ROS contributes to albuminuria in diabetic nephropathy, whereas administration of BH4 ameliorates glomerular NO/ROS balance.16 We also reported that angiotensin receptor blockers ameliorated eNOS uncoupling by improving the levels of guanosine triphosphate cyclohydrolase I (GTPCH I), the rate-limiting enzyme in BH4 biosynthesis.17 Hence, administration of BH4 or activation of BH4 synthesis may be useful in the amelioration of eNOS dysfunction. However, no studies have been performed to examine the effectiveness of BH4 in diabetic nephropathy. BH4 is synthesized from guanosine triphosphate in endothelial cells in a three-step pathway. GTPCH I, encoded by GCH1, is the first and rate-limiting enzyme in the de novo synthesis of BH4.18 In diabetes, GTPCH I degradation is accelerated in a proteasome-dependent manner.19 Therefore, BH4 deficiency due to oxidation and acceleration of GTPCH I degradation is closely associated with nitric oxide synthase (NOS) dysfunction in diabetic vascular complications. However, the role of intrarenal GTPCH I and its molecular pathway in diabetic nephropathy have yet to be elucidated.

We hypothesized that preservation of GTPCH I expression and improvement of NO availability can prevent diabetic nephropathy. To verify this hypothesis, we crossed mice overexpressing human GTPCH I targeted to the vascular endothelium under the control of Tie-2 promoter (GCHtg)20 with spontaneously diabetic Ins2Akita (Ins2+/Akita) mice21 and investigated the relationship between BH4–eNOS coupling and glomerular permeability to albuminuria. In addition, recent reports indicate that AMP-activated protein kinase (AMPK) suppresses proteasome-dependent protein degradation in vitro.22 Moreover, some reports show that proteasome-dependent GTPCH I degradation is key for diabetes-induced endothelial dysfunction.19,23 Therefore, we also investigated whether metformin, an oral hypoglycemic agent and AMPK activator, exerts a protective effect in diabetic nephropathy.

RESULTS

Physiologic and Biochemical Data

Male C57BL/6 mice (WT) mice, GCHtg mice, Ins2+/Akita mice, and GCHtg/Ins2+/Akita mice were compared at 15 weeks of age. Physiologic characteristics of the four groups of mice are listed in Table 1. Body weight and the serum creatinine levels in Ins2+/Akita mice and GCHtg/Ins2+/Akita mice were significantly lower than in WT and GCHtg mice. There was no significant difference among the groups with respect to BP. Serum glucose and kidney per body weight ratio in Ins2+/Akita and GCHtg/Ins2+/Akita mice were increased compared with that in WT and GCHtg mice. The urinary albumin levels in Ins2+/Akita mice were significantly higher than that in WT and GCHtg mice. However, the urinary albumin levels in GCHtg/Ins2+/Akita mice were significantly lower than in Ins2+/Akita mice.

Glomerular Morphologic Change and Immunostaining of α-Smooth Muscle Actin and Podocin

Glomerular size (entire perimeter) in Ins2+/Akita and GCHtg/Ins2+/Akita mice was increased compared with WT mice (Figure 1A and B). Moreover, the glomerular collagen IV–positive area, which indicates the glomerular mesangial matrix, was larger in Ins2+/Akita mice than in WT mice but was smaller in GCHtg/Ins2+/Akita mice than in Ins2+/Akita mice (Figure 1, A and C). Morphologic changes in glomerular endothelium were examined by scanning and transmission electron microscopy (Figure 1D). Glomerular endothelial cells (GECs) are highly specialized cells with regions of attenuated cytoplasm punctuated by numerous fenestrae. Absence of these structures was observed in Ins2+/Akita mice; however, fenestration was maintained in GCHtg/Ins2+/Akita mice (Figure 1D). The α-smooth muscle actin (α-SMA)—immunosstained area in Ins2+/Akita mice was larger than that in WT and GCHtg/Ins2+/Akita mice (Figure 1E and F). Podocin, a slit membrane protein, was detected in podocytes of the glomerular capillaries in immunohistochemically stained sections of mice in each group. Quantitative analysis

Table 1. Physiologic and biochemical data

<table>
<thead>
<tr>
<th>Group</th>
<th>WT</th>
<th>GCHtg</th>
<th>Ins2+/Akita</th>
<th>GCHtg/Ins2+/Akita</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>30.1±1.6</td>
<td>33.5±1.8</td>
<td>25.4±0.7ab</td>
<td>24.5±0.3ab</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>110±8</td>
<td>112±5</td>
<td>108±6</td>
<td>109±4</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>67±9</td>
<td>70±3</td>
<td>59±3</td>
<td>61±4</td>
</tr>
<tr>
<td>Kidney weight (left kidney weight/body weight)</td>
<td>0.72±0.05</td>
<td>0.62±0.03</td>
<td>1.22±0.05ab</td>
<td>1.25±0.04ab</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>225±9</td>
<td>190±9</td>
<td>623±47ab</td>
<td>585±24ab</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.16±0.01</td>
<td>0.18±0.01</td>
<td>0.14±0.01ab</td>
<td>0.14±0.01ab</td>
</tr>
<tr>
<td>Urinary albumin excretion (µg/dl)</td>
<td>78±9</td>
<td>24±2a</td>
<td>401±56ab</td>
<td>226±39abc</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

aP<0.05 versus WT.

bP<0.05 versus GCHtg.

cP<0.05 versus Ins2+/Akita.
showed no significant differences in the podocin-stained area among the groups (Figure 1, G and H).

**In Situ Detection of ROS/NO Production and Assessment of O$_2^{-}$ Production and eNOS Uncoupling in Mouse Kidney**

The GCHtg and GCHtg/Ins2$^{+/Akita}$ mice showed significantly higher levels of total biopterin (BH4+7,8-dihydrobiopterin [BH2]) and BH4 than did the WT and Ins2$^{+/Akita}$ mice (Figure 2A). There was no significant difference in the levels of total biopterin and BH4 in the WT and Ins2$^{+/Akita}$ mice (Figure 2A). However, the BH4/BH2 ratio in Ins2$^{+/Akita}$ mice was significantly decreased compared with that in other groups (Figure 2B). The production of ROS and NO in the kidney tissue was evaluated by confocal laser microscopy using 2',7'-dichlorofluorescein-diacetate and diaminorhodamine-4M acetoxymethyl ester with l-arginine (Figure 2C). Increased ROS production and diminished NO bioavailability were noted in the glomeruli of...
Ins2+/Akita mice but were significantly ameliorated in GCHtg/Ins2+/Akita mice, indicating preserved eNOS coupling. Basal O$_2^-$ production, measured using lucigenin-enhanced chemiluminescence of whole kidney, was significantly decreased in GCHtg/Ins2+/Akita mice compared with that in Ins2+/Akita mice (Figure 2D). NOS inhibition by NG-nitro-l-arginine methyl ester resulted in a significant decrease in chemiluminescence in Ins2+/Akita mice and negligible change in GCHtg/Ins2+/Akita mice, which indicating preserved eNOS coupling by GTPCH I in GCHtg/Ins2+/Akita mice (Figure 2D). Stimulation with NAD(P)H increased O$_2^-$ production in both groups dramatically (Figure 2E), but these levels remained significantly lower in GCHtg/Ins2+/Akita mice compared with that in Ins2+/Akita mice.

Evaluation of Glomerular Endothelial Surface Layer
The glomerular endothelial surface layer (ESL) was detected using wheat germ agglutinin (WGA) lectin staining (Figure 3, A–C). The WGA-stained area on the capillary wall was significantly smaller in Ins2+/Akita mice (Figure 3B) compared with that in WT mice (Figure 3A) but was significantly ameliorated in GCHtg/Ins2+/Akita mice (Figure 3C). Figure 3D provides a graph of the semi-quantitative analysis of the fluorescence intensity.

Examination of Glomerular Capillary Permeability of Macromolecules
Glomerular capillary permeability of macromolecules was visualized using intravenous injection of FITC-conjugated dextran, as previously reported. A very small volume of filtered 70-kD FITC-labeled dextran was detected in the Bowman’s capsule space of WT mice (Figure 4A and Supplemental Video 1) but a massive leakage of this dextran was seen in Ins2+/Akita mice (Figure 4B and Supplemental Video 2). The leakage level of this dextran, however, was ameliorated in GCHtg/Ins2+/Akita mice (Figure 4C and Supplemental Video 3).

Physiologic and Biochemical Data in WT/Met and Ins2+/Akita/Met mice
The physiologic characteristics of the four groups of mice are listed in Table 2. Body weight and serum creatinine levels in Ins2+/Akita and Ins2+/Akita/Met mice were significantly lower
than in WT mice. Urinary albumin levels in Ins2+/Akita mice were significantly higher than in WT and WT/Met mice. However, the urinary albumin levels in Ins2+/Akita/Met mice were significantly lower than in Ins2+/Akita mice, with no alteration in blood glucose level and BP.

Biopterin Level in Mouse Kidney Treated with Metformin
The BH4 level was significantly higher in WT/Met and Ins2+/Akita/Met mice compared with WT and Ins2+/Akita mice (Table 3), whereas there was no significant difference in the BH4 levels in WT and Ins2+/Akita mice. However, the BH4/BH2 ratio in Ins2+/Akita mice was significantly lower than that in the other groups.

Evaluation of the Levels of GTPCH I mRNA and Protein and AMPK Activity in Mouse Kidney Treated with Metformin
The expression of mouse GTPCH I mRNA in Ins2+/Akita and Ins2+/Akita/Met mice was significantly higher than in WT and WT/Met mice (Figure 5A). As previously indicated, Ins2+/Akita mice showed significantly lower GTPCH I protein levels than did WT mice (Figure 5B). However, GTPCH I protein levels in Ins2+/Akita mice treated with metformin was higher than that in Ins2+/Akita mice (Figure 5B). The phosphorylated AMPK staining of glomerular sections in Ins2+/Akita mice was decreased compared with that in WT and WT/Met mice (Figure 5C). Ins2+/Akita/Met mice showed a higher level of phosphorylated AMPK staining in glomeruli than did Ins2+/Akita mice (Figure 5C).

Evaluation of Biopterin Levels and eNOS Dimer/Monomer Structures in Human GEnCs
The level of total biopterin and BH4 was nearly identical in all groups. However, the BH4 level in GEnCs cultured in high glucose (HG) showed a downward tendency and was significantly lower than that in GEnCs cultured in HG and metformin (HG/Met) (Figure 6A). The BH4/BH2 ratio in HG GEnCs was significantly lower than that in the other groups (Figure 6B). Furthermore, the eNOS dimer/monomer ratio in HG GEnCs was significantly lower than that in GEnCs cultured in normal glucose; however, treatment with metformin ameliorated this ratio (Figure 6C).

Evaluation of AMPK Activation, GTPCH I Level, and 26S Proteasome Activity of GEnCs in the Presence or Absence of Metformin/AICAR
We analyzed the effects of metformin on AMPK activation (Figure 7A) and GTPCH I expression (Figure 7B). HG concentration reduced the levels of AMPK-Thr172 phosphorylation, the active form of AMPK, and GTPCH I in human GEnCs; however, treatment with metformin attenuated these effects. In addition, we examined AMPK activation and GTPCH I level using 5-aminomidazole-4-carboxamide riboside (AICAR), an AMPK activator. Similar to metformin, AICAR attenuated the reduction of AMPK activity and GTPCH I level in GEnCs (Figure 7C). The levels of GTPCH I protein are controlled by both GTPCH I synthesis and GTPCH I degradation. HG activates 26S proteasome, resulting in enhanced degradation of GTPCH I in endothelial cells. We evaluated 26S proteasome activity induced by high glucose stimulation and the effect of metformin. HG significantly increased 26S proteasome activity in human GEnCs. Metformin suppressed 26S proteasome activity induced by HG in human GEnCs (Figure 7D). Coadministration of MG132 (0.5 µM), a potent proteasome inhibitor, abolished the HG-induced activation of 26S proteasome (data not shown). Furthermore, GTPCH I protein level increased in both glucose conditions after preincubation of MG132 (Figure 7B). The expression of human GTPCH I mRNA was increased in HG compared with normal glucose. Metformin was not involved in the regulation of GTPCH I mRNA expression in GEnCs (Figure 7E).

DISCUSSION
This study examined whether maintaining the intrarenal levels of GTPCH I and BH4 inhibited the progression of diabetic nephropathy and whether the intrarenal levels of these biomolecules could be ameliorated by metformin, which is an AMPK activator. Intrarenal GTPCH I level was decreased in mice with diabetic nephropathy but was increased in mice
overexpressing GTPCH I. Higher levels of GTPCH I and BH4 reduced glomerular ROS production and improved glomerular hyperpermeability and urinary albumin excretion. Metformin has been suggested to inhibit degradation of renal GTPCH I and to exert a renal protective effect. Collectively, these results show that reducing levels of intrarenal GTPCH I is profoundly involved in the progression of diabetic nephropathy, which is recognized as a manifestation of diabetic microangiopathy, and that maintaining GTPCH I activity might delay the progression of this condition.

Figure 4. Examination of glomerular capillary permeability of macromolecules. Representative series of images from the WT (A), Ins2+/Akita (B), and GCHtg/Ins2+/Akita (C) showing hyperfiltration of macromolecules in glomeruli per mouse group as indicated. Green, 70-kD FITC-labeled dextran. Time per frame, 870 ms. Bar, 40 μm.

Table 2. Physiologic and biochemical data

<table>
<thead>
<tr>
<th>Group</th>
<th>WT</th>
<th>WT/Met</th>
<th>Ins2+/Akita</th>
<th>Ins2+/Akita/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>30.1±1.6</td>
<td>30.5±1.6</td>
<td>25.4±0.7(^a)(^b)</td>
<td>24.6±0.5(^a)(^b)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>103±2</td>
<td>102±2</td>
<td>108±3</td>
<td>109±4</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>63±3</td>
<td>57±3</td>
<td>59±2</td>
<td>55±4</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.16±0.01</td>
<td>0.14±0.01(^a)</td>
<td>0.14±0.01(^a)</td>
<td>0.11±0.01(^a)(^b)(^c)</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>225±9</td>
<td>180±13(^a)</td>
<td>622±47(^a)</td>
<td>633±30(^a)</td>
</tr>
<tr>
<td>Urinary albumin excretion (μg/dl)</td>
<td>100±17</td>
<td>67±16</td>
<td>387±46(^a)(^b)</td>
<td>225±43(^a)(^b)(^c)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

\(^a\)P<0.05 versus WT.

\(^b\)P<0.05 versus WT/Met.

\(^c\)P<0.05 versus Ins2+/Akita.

for the NOS isoforms, including eNOS. BH4 is converted to BH2 under oxidative stress. Decreased levels of BH4 result in an eNOS dysfunction known as eNOS uncoupling. Uncoupled eNOS produces more ROS than NO.\(^{14}\)

Alp et al. reported that both the BH4 level and the BH4/BH2 ratio are decreased in the aorta of apoE-knockout mice.\(^{25}\) Some reports have shown that the BH4/BH2 ratio regulates eNOS function independent of the BH4 level.\(^{26,27}\) d’Uscio et al. reported that the BH4/BH2 ratio is significantly decreased in the liver of apoE-knockout mice although the BH4 level is not significantly distorted.\(^{27}\) Our results suggest that a lower BH4/BH2 ratio is strongly associated with formation of uncoupled eNOS, and increased production of BH4 by GTPCH I overexpression induces eNOS recoupling, which
attenuates oxidative stress and leads to oxidation of BH4 in diabetic states.

We found that renal BH4 level and BH4/BH2 ratio were increased in GCHtg/Ins2\(^{+/Akita}\) mice compared with Ins2\(^{+/Akita}\) mice. eNOS coupling levels are also higher in GCHtg/Ins2\(^{+/Akita}\)-Tg mice compared with that in Ins2\(^{+/Akita}\) mice. Ali et al. showed that oxidative stress was attenuated by maintaining vascular BH4 level by suppressing eNOS in apoE-knockout mice crossed with tie2-GCHtg mice.28 Du et al. reported that vascular oxidative stress was attenuated in hypertensive rats crossed with tie2-GCHtg mice.29 Similar to the findings of these reports, our results indicated that the maintenance of renal BH4 level and BH4/BH2 ratio suppressed oxidative stress and protected the kidney against diabetic nephropathy.

In this study, we showed that eNOS recoupling decreased glomerular oxidative stress by maintaining the BH4 level, which in turn ameliorated glomerular hyperfiltration and vascular permeability through the preservation of glyocalyx, a major component of the ESL. The glomerular filtration barrier is composed of GEnCs, a glomerular basement membrane, and glomerular epithelial cells. It is believed that GEnCs do not play an important role in the filtration barrier because of the many small pores known as fenestrae, which are 60–80 nm in diameter. It has recently been reported that GEnCs are covered by an ESL, containing glyocalyx and cell coat, which extends into the fenestrae.30,31 The ESL covers the cellular surface and is composed of large amounts of glycoprotein, such as heparan sulfate proteoglycan.32 Because of the presence of sulfated sugar chains in heparan sulfate proteoglycan, the ESL possesses a highly negative charge and thereby regulates vascular permeability. A decrease in the thickness of the ESL is associated with an increase in vascular permeability and the onset of albuminuria. We have demonstrated that the glomerular ESL is implicated in the regulation of glomerular wall permeability and that ROS-induced deterioration of the ESL exacerbates glomerular permeability in Zucker fatty rats.24 Salmon et al. reported that loss of endothelial glyocalyx is associated to albuminuria with vascular dysfunction.25 Furthermore, Gil et al. reported that the deletion of the heparanase gene, the predominant enzyme that degrades heparan sulfate, protects diabetic mice from diabetic nephropathy.34 Production of heparanase has been shown to be regulated by oxidative stress.35 It is considered that the improvement of glomerular permeability by preservation of glyocalyx in GCHtg/Ins2\(^{+/Akita}\) mice results from inhibiting ROS production via eNOS recoupling.

We have shown that urinary albumin excretion was decreased and intrarenal GTPCH I level and BH4/BH2 ratio were ameliorated in diabetic mice that were administered metformin. In a clinical trial, it was shown that metformin has beneficial effects on cardiovascular diseases in type 2 diabetes.36 More recently, it was demonstrated that the target molecule of metformin is AMPK.37 Moreover, it was shown that AMPK suppresses proteasome-dependent proteolysis in vitro.22 Xu et al. reported that proteasome-dependent degradation of GTPCH I was related to endothelial dysfunction in diabetes.19 Wang et al. reported that the inhibition of GTPCH I degradation by AMPK activation attenuated endothelial dysfunction.23 Our data showed that metformin exerts a protective effect on GEnCs as well as vascular endothelial cells via maintenance of GTPCH I level. HG-induced overexpression of GTPCH I mRNA on GEnCs. Because metformin did not affect the expression of GTPCH I mRNA on GEnCs in each glucose condition, the protective effect of endothelial cells by metformin would be strongly dependent on inhibition of GTPCH I protein degradation through AMPK activation. Moreover, because AICAR also maintained GTPCH I level via AMPK activation, it was demonstrated that AMPK was important target molecule of metformin; however, there is no evidence that this protective effect extends to the kidneys. There are no reported clinical studies on metformin that established a primary endpoint of urinary albumin excretion. Moreover, metformin has been shown to activate AMPK in a dose-dependent manner in vitro.37 The dosage of metformin used in this study was higher that the dosage used in humans, which might have led to the improvement of endothelial dysfunction.

A limitation of this study is that our findings do not provide enough information on whether type 2 diabetes is improved by supplementation of BH4 via GTPCH I overexpression or administration of metformin. The Ins2\(^{+/Akita}\) mouse used in this study is a type 1 diabetic mouse model. Hence, we need to identify whether these interventions would be effective in type 2 diabetic models. Cheng et al. reported that HG induced GEnCs dysfunction, such as hyperpermeability, apoptosis, and eNOS uncoupling, and these abnormalities ameliorated by coincubation with L-arginine or sepiapterin. Furthermore, they demonstrated that supplementation of L-arginine or

### Table 3. Biopterin level in mouse kidney treated with metformin

<table>
<thead>
<tr>
<th>Group</th>
<th>WT</th>
<th>WT/Met</th>
<th>Ins2(^{+/Akita})</th>
<th>Ins2(^{+/Akita})/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH2 (pmol/mg protein)</td>
<td>0.78±0.03</td>
<td>2.10±0.44(^{a})</td>
<td>1.32±0.10(^{b})</td>
<td>1.40±0.17(^{a,b})</td>
</tr>
<tr>
<td>BH4 (pmol/mg protein)</td>
<td>4.29±0.56</td>
<td>17.22±1.39(^{a,c})</td>
<td>4.02±0.28(^{b})</td>
<td>9.30±1.27(^{a,c})</td>
</tr>
<tr>
<td>BH4/BH2 ratio</td>
<td>5.5±1.0</td>
<td>8.2±1.5(^{a})</td>
<td>3.1±0.2(^{a,b})</td>
<td>6.6±0.2(^{c})</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

\(^{a}\)P<0.05 versus WT.

\(^{b}\)P<0.05 versus WT/Met.

\(^{c}\)P<0.05 versus Ins2\(^{+/Akita}\).
sepiapterin attenuated albuminuria and glomerular basement membrane thickness and decreased oxidative stress in db/db mice, known as type 2 diabetic model mice. This report suggests that improvement of eNOS function might prevent progression of type 2 diabetic nephropathy. Furthermore, we only assessed the effects of our intervention in the early phases of diabetic nephropathy and not progressive diabetic nephropathy.

In conclusion, our data indicated that increased endothelial GTPCH I expression and BH4 biosynthesis by metformin induces recoupling of endothelial nitric oxide synthase and reduces albuminuria in mice with diabetic nephropathy. Further examination is needed to evaluate the suppressive effects of metformin in the progression of diabetic nephropathy.

CONCISE METHODS

Experimental Materials
The experimental protocol (no. 10-031, 12-042) was approved by the Ethics Review Committee for Animal Experimentation at Kawasaki Medical School, Kurashiki, Japan. WT and Ins2+/Akita mice were purchased from Japan SLC (Shizuoka, Japan). GCHtg mice were kindly provided by Keith M. Channon (University of Oxford, John Radcliffe Hospital, Oxford, UK).20 GCHtg mice were crossed with Ins2+/Akita mice to generate GCHtg/Ins2+/Akita mice. Antibodies against AMPK (#2532), and phospho-AMPK (#2531) were from Cell Signaling Technology (Danvers, MA). Antibodies against GCH-1 (sc-100749) and podocin (sc-21009) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against α-SMA, β-catenin, and metformin were from Sigma-Aldrich Japan (Tokyo, Japan). Antibodies against eNOS were from BD Biosciences (San Jose, CA).

In Vivo Experimental Protocol 1
Male WT mice, GCHtg mice, Ins2+/Akita mice, and GCHtg/Ins2+/Akita mice (n=10 each) were housed in a temperature- and humidity-controlled room with a 12-hour light–dark cycle; they were fed standard laboratory animal chow and had free access to tap water. At 15 weeks of age, body weight was recorded, and BP was measured by the tail-cuff method with an automatic sphygmomanometer (BP98A; Softron, Tokyo, Japan). The mice were then placed in metabolic cages for 24 hours to collect urine and were finally sacrificed under sevoflurane inhalation anesthesia for collection of kidney tissue and blood samples. Serum creatinine level was measured using an enzyme assay (Nescoat VLII CRE Kit; Alfresa Pharma, Osaka, Japan). Urinary albumin levels were determined by ELISA using a murine microalbuminuria ELISA kit (AlbuwellM; Exocell, Philadelphia, PA).

In Vivo Experimental Protocol 2
WT mice and Ins2+/Akita mice (each n=8) were treated with metformin (300 mg/kg per day), an oral hypoglycemic drug and a potent AMPK activator, by gavage for 4 weeks from 10 weeks of age. Body weight and BP measurements and urine collection were performed using the methods from experimental protocol 1. After collection of urine, the mice were sacrificed under sevoflurane inhalation anesthesia for collection of kidney tissue and blood samples.

Cell Culture
Primary normal human GEnCs were purchased from Cell Systems (Kirkland, WA) and cultured according to the manufacturer’s instructions.
instructions. Human GEnCs were cultured in endothelial cell basal medium-2 (Lonza, Walkersville, MD) containing v/v 5% FBS under humidified conditions (95% air, 5% CO2) at 37°C. Confluent cells from 7 to 10 passages were used in the experiments. These cells were exposed to 5 mM D-glucose or 30 mM D-glucose in the presence or absence of 2 mM metformin or 2 mM AICAR. These cells were then incubated at 37°C for 24 hours.

**Histologic Assessment**

Sections (4-μm thick) were prepared from renal tissue samples embedded in paraffin and stained with periodic acid–Schiff and tetramethyl rhodamine isothiocyanate-conjugated WGA (Vector Laboratories, Burlingame, CA). Kidney sections were photographed and digitized into color images by using a Nikon Coolscope (Nikon, Tokyo, Japan). Tetramethylrhodamine isothiocyanate-conjugated WGA-stained kidney sections were analyzed using TCS-NT system software (Leica-Microsystems, Tokyo, Japan). Histologic scores were assessed with a Coolscope (Nikon). For the evaluation of glomerular ESL, the percent positive area was measured using a color image analyzer (WinLoof; Mitani Co., Fukui, Japan). A minimum of 100 glomeruli at ×400 magnification were randomly selected from each mouse, and the mean score was calculated.

**Immunohistochemistry**

Cryostat sections (3-μm thickness) were used for immunohistochemical studies of collagen IV GTPCH I, α-SMA, podocin, and phospho-AMPKα (Thr172). Antibody binding was detected using fluorescein-labeled secondary antibodies (for phospho-AMPK) or the Histofine Simple Stain MAX-PO kit and diaminobenzidine (for collagen IV, GTPCH I, and podocin) (Sigma-Aldrich Japan). For evaluation of α-SMA- and podocin-positive areas, the percent positive area was measured using a color image analyzer (WinLoof; Mitani Co., Fukui, Japan). A minimum of 100 glomeruli at ×400 magnification were randomly selected from each mouse, and the mean score was calculated.

**Western Immunoblotting**

Extraction of total cellular protein was performed using an extraction reagent (T-PER Tissue Protein Extraction Reagent; Thermo Fisher Scientific, Rockford, IL), according to the manufacturer’s instructions, and then SDS-PAGE was performed (30–50 μg protein/lane). For immunoblot analysis of the dimeric form of eNOS, samples were not heated and the temperature of the gel was maintained below 15°C during electrophoresis (low-temperature SDS-PAGE).39,40 Furthermore, we added disuccinimidyl suberate, a chemical bivalency cross-linking agent, to the lysis buffer to facilitate the identification of the dimer eNOS form. Anti-GTPCH I antibodies, anti-AMPK antibodies, anti-phospho-AMPK antibodies, and anti-eNOS antibodies were used as the primary antibodies. Signals were detected with the ECL system. Relative optical densities of the bands were quantified with Image J software version 1.42 (http://rsbweb.nih.gov/ij/).

**Quantitative Real-Time PCR**

Total RNA extraction from kidney and human GEnCs samples, reverse transcription reaction, and real-time quantitative PCR were
Figure 7. Evaluation of p-AMPK/AMPK, GTPCH I expression, and 26S proteasome activity in human GEnCs. (A) Western blot analysis for total AMPK and phospho-AMPK. The results are expressed as the phospho-AMPK/AMPK ratio. (B) Western blot analysis for GTPCH I. (C) Western blot analysis for phospho-AMPK/AMPK ratio and GTPCH I using AICAR. (D) 26S proteasome activity was measured using fluorescent proteasome substrates. (E) Expression of human GTPCH I mRNA. Data are expressed as mean ± SEM. *P<0.05 versus NG/Met–; †P<0.05 versus NG/Met+; ‡P<0.05 versus HG/Met–.
performed as previously described. The primers and probe for human GCH1 (NM_000161) were as follows: forward, agtcatggagccctttaca; reverse, tcacacctaccccgact; and probe, agccaatggtcctgcggcc. The primers and probe for mouse GCH1 (NM_008102) were as follows: forward, caaggctacaggaggaca; reverse, agcaatggtcctgcggcc; and probe, cagcgtgagccatctctg. The primers and probes were designed using Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and confirmed by Amplify 3 software version 3.1.4 (http://engels.genetics.wisc.edu/amplify/).

**Determination of Renal BH4 Concentrations**

BH4 concentrations were determined by high-performance liquid chromatography as previously described. The amount of BH4 was determined from the difference between total biopterin and alkaline-stable oxidized biopterin. The AC18 column (length, 250 mm; inner diameter, 4.6 mm; particle size, 5 μm) was used with 15 mmol/L K2HPO4 buffer, pH 6.0, at a flow rate of 0.8 ml/min. BH4 was detected by emission fluorescence at 350 (excitation) and 440 nm (emission).

**In Situ Detection of NO and ROS**

The production levels of NO and ROS resulting from NOS coupling were imaged by confocal laser microscopy after renal perfusion using 2′,7′-dichlorofluorescein-diacetate and diaminorhodamine-4M acetoxymethyl ester (excitation/emission maxima, 560/575 nm; Sekisui Medical, Tokyo, Japan) with L-arginine as previously described.

**Lucigenin Chemiluminescence Assay for Measurement of NAD(P)H Oxidase Activity**

NAD(P)H oxidase activity in the kidney was measured using lucigenin chemiluminescence, as previously described. Chemiluminescence was measured using 5 μM lucigenin under basal conditions, after addition of 100 μM NADPH and 1 mM Nε-nitro-l-arginine methyl ester. Lucigenin chemiluminescence was expressed in units per min (unit). In vivo imaging of fluorochrome-labeled albumin was performed by intravenous injection of FITC-dextran (anionic; excitation/emission maxima, 494/518 nm) was used with 15 mmol/L K2HPO4 buffer, pH 6.0, at a flow rate of 0.8 ml/min. BH4 was detected by emission fluorescence at 350 (excitation) and 440 nm (emission).

**In Vivo Imaging of Macromolecule Hyperfiltration**

For fluorescent probes, 70 kDa fluorescein–dextran (ammonic; excitation/emission maxima, 494/518 nm) was obtained from Invitrogen (Tokyo, Japan). A multiphoton excitation laser scanning fluorescence microscopy confocal microscope system (TCS SP2 AOBS MP; Leica Microsystems) was used. In vivo imaging of the glomerular microcirculation was performed, as previously described. For the analysis of glomerular permeability, a 70-kDa fluorescein–dextran solution was infused through the jugular venous catheter.

**26S Proteasome Activity Assay**

The 26S proteasome function was measured, as previously described.

**Statistical Analyses**

Values are expressed as mean ± SEM. Statistical comparisons were made using the Mann–Whitney U test or the one-factor ANOVA with a Tukey–Kramer test for multiple comparisons. A P value <0.05 was considered significant.

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**DISCLOSURES**

None.

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