Bilirubin and biliverdin protect rodents against diabetic nephropathy by downregulating NAD(P)H oxidase

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Bilirubin and Biliverdin Protect Against Diabetic Nephropathy via Down-regulation of NAD(P)H Oxidase in Rodents

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Running title: Bilirubin/Biliverdin ameliorate DM nephropathy
ABSTRACT

We recently reported a markedly lower prevalence of vascular complications, including nephropathy, in diabetic patients with Gilbert syndrome, a congenital hyperbilirubinemia, implicating the beneficial effect of bilirubin on diabetic nephropathy. To test this hypothesis, we investigated whether hereditary hyperbilirubinemic Gunn j/j rats and biliverdin-treated diabetic db/db mice are resistant to the development of renal dysfunction after the onset of diabetes. Both diabetic Gunn j/j rats and biliverdin-treated db/db mice exhibited less albuminuria and complete protection against the progression of mesangial expansion accompanied by normalization of TGF-β1 and fibronectin expression. These effects were paralleled with normalization of urinary and renal oxidative stress markers, renal expression of NAD(P)H oxidase subunits. In cultured vascular endothelial cells and mesangial cells, bilirubin and biliverdin significantly inhibited NADPH-dependent superoxide production and inhibited both high glucose-induced and angiotensin II-induced reactive oxygen species (ROS) production. Collectively, these findings suggest that bilirubin and biliverdin may protect against diabetic nephropathy, at least in part by inhibiting renal NAD(P)H oxidase-derived ROS production. These findings may lead to novel antioxidant therapies for diabetic nephropathy.
INTRODUCTION

Diabetic nephropathy is a leading cause of end-stage renal failure worldwide. Recent epidemiological studies have revealed that diabetic nephropathy is also associated with increased risk of cardiovascular events. A therapeutic approach targeting its causative mechanisms urgently needs to be established. In recent years, oxidative stress has been considered to be an important pathogenic factor in the development of diabetic vascular complications, including nephropathy [1] [2] [3] [4]. Accumulating evidence has shown that many protein, lipid and DNA markers of oxidative stress are increased in kidney and vascular tissues from animals and patients with diabetes[3] [4, 5]. However, trials using various known antioxidants failed to protect against diabetic nephropathy in humans.

Bilirubin(BIL) has been recognized as an endogenous antioxidant for many years[6]. Its formation is mediated by the ubiquitously expressed heme oxygenase (HO), the rate-limiting enzyme involved in heme catabolism. HO participates in heme breakdown to generate biliverdin(BVD), free ferrous iron and carbon monooxide. Subsequently, BVD is rapidly converted to BIL by BVD reductase. Recently, increasing evidence has suggested that HO and its reaction product BIL may serve as important endogenous agents with cytoprotective activity against oxidative stress injury[7-9]. The anti-oxidative action of BIL has been attributed to its highly efficient radical scavenging effect[6], whereas recent reports have shown that its hydrophobic tetrapyrrole structure may inhibit the activity of NAD(P)H oxidase[10, 11]. Notably, we and other investigators have shown that the activation of NAD(P)H oxidase may be a major cause of increased oxidative stress in diabetic renal and vascular tissues[12-15]. Therefore, we
speculated that BIL might have a beneficial effect on diabetic nephropathy. In this context, we recently reported a markedly lower prevalence of nephropathy and other vascular complications in diabetic patients with Gilbert syndrome, a congenital hyperbilirubinemia, along with reduced markers of oxidative stress and inflammation[16]. However, this association study does not necessarily implicate BIL as causative in the observed lower prevalence of diabetic nephropathy. Therefore, to test this hypothesis and explore the underlying molecular mechanisms, we investigated 1) whether hereditary hyperbilirubinemic Gunn rats are resistant to the progression of nephropathy after the onset of diabetes, 2) whether administration of BVD, the precursor of BIL, protects against diabetic nephropathy in db/db mice, a rodent model of type 2 diabetes. The underlying molecular mechanisms were also explored in these experimental animal models and in vitro culture studies.

RESULTS

Hyperbilirubinemic Gunn rats

The characteristics of experimental rats at 0, 8 and 24 weeks after onset of diabetes are shown in Table 1. The total BIL levels remained constant after age of 4 weeks in Gunn j/j rats and Gunn j/+ rats. At 8 weeks after the onset of diabetes, the total serum BIL levels were 0.15±0.02 and 0.18±0.04 mg/dl in diabetic and non-diabetic Gunn j/+ rats, respectively, and 7.01±0.43 and 9.47±0.04 mg/dl in diabetic and non-diabetic Gunn j/j rats, respectively (Table.1). Such marked increments in serum BIL levels in Gunn j/j rats reflect increases in the levels of unconjugated BIL.
Diabetic Gunn j/+ rats exhibited marked increases in the amounts of urinary albumin excretion compared with non-diabetic Gunn j/+ rats at 8 weeks (P<0.01) and 24 weeks (P<0.001) after the onset of diabetes, whereas diabetic Gunn j/j rats exhibited significantly less urinary albumin excretion than diabetic Gunn j/+ rats at 8 weeks (P<0.05) and 24 weeks (P<0.001) (Fig. 1).

To explore the mechanism underlying these beneficial effects of hyperbilirubinemia, we measured systemic oxidative stress markers, urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) excretion and 8-epi-prostaglandin (PG) F\textsubscript{2α} levels. Urinary excretion levels of both 8-OHdG and 8-epi-PG F\textsubscript{2α} were significantly higher in diabetic Gunn j/+ rats than in non-diabetic Gunn j/+ rats at 8 weeks. (Fig. 2a, b). The diabetes-induced increases in both oxidative markers at 8 weeks were completely prevented in diabetic hyperbilirubinemic Gunn j/j rats, which showed levels comparable to those in non-diabetic Gunn j/+ rats.

Immunostaining analysis of 8-OHdG in renal tissues revealed that the staining intensities in diabetic Gunn j/+ rats were significantly higher than those in control Gunn j/+ rats, in both glomeruli and tubules at 24 weeks (Fig. 2c-f). These increases in 8-OHdG staining intensities in glomeruli and tubules were completely prevented in diabetic Gunn j/j rats, which showed levels comparable to those in control Gunn j/+ rats (both non-significant vs. control).

Furthermore, we examined the expression of renal NAD(P)H oxidase components. To examine the localization and expression levels of NOX4 protein, we carried out immunostaining analysis. The staining intensities for NOX4 protein were stronger in the renal glomeruli and tubules of diabetic Gunn j/+ rats than in those of control Gunn j/+ rats (Fig 3a, b). Western blotting analysis confirmed that the protein levels for NOX4 were significantly increased in the
kidneys of diabetic Gunn j/+ rats compared with control Gunn j/+ rats (P<0.001) (Fig. 3c). All of these changes were completely prevented in diabetic Gunn j/j rats, which showed levels comparable to those in control Gunn j/+ rats.

Real-time PCR analysis showed that the mRNA levels for NOX4 and other components, p22phox and p47phox, were also significantly increased in the kidneys of diabetic Gunn j/+ rats compared with control Gunn j/+ rats (Fig. 3d-f). All of these changes were completely prevented in diabetic Gunn j/j rats, which showed levels comparable to those in control Gunn j/+ rats.

We investigated the effect of hyperbilirubinemia on mesangial expansion at 24 weeks after the onset of diabetes. The glomerular structure in diabetic Gunn j/+ rats showed accelerated mesangial expansion compared with that observed in control Gunn j/+ rats (Fig. 4a). The PAS-positive and nuclei-free mesangial area was markedly increased in the glomeruli of diabetic Gunn j/+ rats (P<0.001) (Fig. 4b). Diabetic Gunn j/j rats showed complete prevention of mesangial expansion.

We also examined the effect of hyperbilirubinemia on the levels of transforming growth factor-β1 (TGF-β1) and fibronectin. The levels of TGF-β1 / fibronectin mRNA and protein were significantly increased in the kidneys of diabetic Gunn j/+ rats compared with control Gunn j/+ rats (Fig. 4 c-f). In diabetic Gunn j/j rats, the increases in TGF-β1 and fibronectin levels were significantly prevented.

**BVD administration**

Next, we investigated the effect of BVD administration on albuminuria, oxidative stress and
renal mesangial expansion in db/db mice.

BVD administration (5mg/kg) orally for 2 weeks and 12 weeks did not significantly affect body weights or blood glucose levels (Table 2). Oral administration of this dose of BVD did not induce a significant elevation in serum BIL levels (Table 2), although an intraperitoneal injection of the same dose induced a slight elevation in serum BIL levels at 0.5 h, with rapid return to the basal levels at 6 h after injection (data not shown).

Urinary albumin excretion significantly increased in non-treated db/db mice as compared with control db/+ mice at 2 weeks and 12 weeks after the start of BVD administration (Fig.5). BVD administration significantly attenuated such increases in urinary albumin excretion in db/db mice.

Each oxidative stress marker (urinary 8-OHdG, 8-epi-PG F_2α excretion and immunostaining analysis of 8-OHdG in renal tissues) was significantly higher in db/db mice than in control db/+ mice. BVD administration completely normalized these markers in db/db mice to control levels (Fig.6a-f). We also confirmed the effect of BVD to intracellular production of superoxide in the renal tissues by dihydroethidium (DHE) stain. The oxidized DHE signals were significantly higher in db/db mice than those in control mice at 12 weeks (Fig. 6g-j). BVD administration completely normalized oxidized DHE signals to the control levels. Then we evaluated the NADPH oxidase activities, using the lucigenin-enhanced chemiluminescence method as previously described [17]. BVD administration significantly suppressed diabetes-induced activation of NADPH oxidase in crude cortex homogenates up to control levels (Fig. 6k).

Furthermore, to examine the levels of NOX4 protein, we carried out immunostaining analysis
and Western blot analysis. The protein levels of NOX4 were also significantly increased in renal tissues of db/db mice. BVD administration completely normalized all of these changes in db/db mice to the control levels (Fig. 7a-c).

The levels of mRNA for NOX4 and other components, p22phox and p47phox, were also significantly increased in the kidney of db/db mice as compared with control mice (P<0.05; P<0.05; P<0.05) (Fig. 7d-f). Likewise, the levels of mRNA for NAD(P)H oxidase components in glomeruli were significantly increased in db/db mice as compared with control mice (P<0.01; P<0.01; P<0.01) (Fig. 7g-i). These abnormalities were completely normalized by BVD administration. We evaluated the levels of mRNA for HO-1 in all these groups, but there was no significant change among them.

Mesangial expansion was found in db/db mice at the age of 24 weeks (P<0.001) (Fig. 8a, b), with increased mRNA and protein levels of TGF-β1 (Fig. 8c, e) and fibronectin (Fig. 8d, f) in the kidney of db/db mice, as well as in glomeruli (Fig. 8g, h). These abnormalities were completely prevented by BVD administration.

In vitro effect of BIL and BVD on NAD(P)H oxidase activities.

The effect of BIL and BVD on NAD(P)H oxidase activities in cultured human mesangial cells were evaluated by the lucigenin method. Pretreatment of the cells with both BIL and BVD for 48 hours reduced NAD(P)H oxidase activities in a dose-dependent manner (Fig. 9a, b). In addition, intracellular oxidative stress was evaluated by the 2', 7'-diclorofluorescein diacetate (DCF-DA) staining method as previously described[18]. DCF-DA staining showed that exposure of the cells
to angiotensin II (Ang II) (100 nM) for 4 h and high glucose levels (450mg/dl) for 24 h increased intracellular oxidative stress as compared to the control levels (Fig. 9c, d). These increases in intracellular oxidative stress were completely normalized to the control levels by treatment with BIL for 1h (10 µM).

Finally, we investigated the effect of BIL and BVD on the expression of NOX4, TGF-β1 and fibronectin in cultured human mesangial cells. Ang II stimulated the expression levels of NOX4 mRNA in a time-dependent manner (Fig.10a). Treatment with BIL and BVD completely inhibited the increased expression of NOX4 mRNA, whereas N-acetylcysteine(NAC) and α-Lipoic acid(α-LA) did not, even at the maximal concentration. The protein levels of NOX4 were also inhibited by BIL and BVD (Fig. 10b). We confirmed that the increased expression of mRNA of TGF-β1 and fibronectin induced by Ang II was also completely prevented by BIL and BVD in all time courses, whereas NAC and α-LA did not (Fig.10c, d).

**DISCUSSION**

In this study, to reveal the protective effect of BIL on diabetic nephropathy, we first used streptozotocin-induced diabetic homozygous Gunn rats, which exhibit a marked elevation of plasma unconjugated BIL levels due to a genetic deficiency of uridine diphosphate glucuronosyl transferase-1 (UDPGT-1). The present study showed that diabetic hyperbilirubinemic Gunn j/j rats exhibited significantly less urinary albumin excretion than diabetic non-hyperbilirubinemic Gunn j/+ rats. In addition, diabetic Gunn j/j rats did not develop renal mesangial expansion, which is one of the most striking morphological features of diabetic nephropathy, 6 months after
the onset of diabetes, whereas diabetic Gunn j/+ rats developed typical mesangial expansion.

TGF-β is a key cytokine that mediates the changes, which leads to extracellular matrix accumulation and glomerular expansion in diabetes [19]. We also found that the increased expression of TGF-β and fibronectin, a major matrix protein, was prevented in diabetic Gunn j/j rats. These findings suggested that hyperbilirubinemic Gunn j/j rats were resistant to the progression of functional and morphological features of nephropathy after the onset of diabetes.

Secondly, we used db/db mice, a rodent model of type 2 diabetes to evaluate the effect of administration of BVD, a precursor of BIL. We administered BVD (5 mg/kg) orally instead of BIL, because BVD is relatively more water-soluble than BIL. Oral administration of this dose of BVD did not induce a significant elevation in serum bilirubin levels. Nevertheless, the present study showed that administration of BVD (5 mg/kg) protected against both albuminuria and renal mesangial expansion in db/db mice. Since serum BVD enters cells rapidly and is converted to BIL by BVD reductase[20], it is likely that the beneficial effect of BVD administration may be due to increased levels of intracellular BIL levels generated from exogenously administered BVD, rather than increased levels of serum BIL or BVD.

The present study further suggested that the mechanism underlying these beneficial effects of hyperbilirubinemia and BVD administration may be the inhibition of oxidative stress, evaluated by systemic oxidative stress markers (urinary excretion of 8-OHdG and 8-epi-PG F2α), 8-OHdG staining, and DHE oxidation levels which are relatively superoxide-sensitive in renal tissues. Since BIL has been recognized to possess radical scavenging activity [6], the inhibition of oxidative stress may be, at least in part, due to its radical scavenging activity. Notably, the serum
BIL levels in diabetic Gunn j/j rats were slightly, but significantly lower than those in non-diabetic Gunn j/j rats. Such a decrease in serum BIL levels induced by diabetes may be a reflection of its consumption owing to its radical scavenging activity, although this speculation should be confirmed in future studies.

However more importantly, we revealed for the first time that biliverdin administration induced down-regulation of NAD(P)H oxidase components (NOX4, p22phox, p47phox) in diabetic kidneys, glomeruli and human mesangium cells. Although the nature of the sources of ROS overproduction in diabetes has not been precisely defined, we and other investigators have indicated that non-phagocytic NAD(P)H oxidases may be the major sources of increased ROS production in the vascular tissues of diabetic animals and patients [12, 13, 15]. The non-phagocytic NAD(P)H oxidase comprises of a membrane-associated cytochrome b558 composed of NOX family proteins (gp91phox, NOX1, NOX4) and p22phox, and several cytosolic regulatory components, p47phox, p67phox and Rac 1 or Rac 2 [21]. The isoform NOX4 was cloned from the kidney, where it was found to be highly expressed [22, 23]. It has been suggested that NOX4, as a major source of ROS production in the kidney, could have a role under pathological conditions. We previously reported that increased expression of NOX4 may play an important role in increased ROS production in the kidneys of streptozotocin-induced diabetic rats[24-26]. Gorin et al. confirmed increased expression of NOX4 in the diabetic kidney, and further revealed that down-regulation of NOX4 induced by antisense oligonucleotides completely attenuated oxidative stress in the kidneys of streptozotocin-induced diabetic rats[27].

Taken together, the present results suggest that down-regulation of NAD(P)H oxidase...
components, especially NOX4, by hyperbilirubinemia and BVD administration may play an
important role in the inhibition of oxidative stress in the kidneys of diabetic rodents. We also
confirmed the inhibitory effect of hyperbilirubinemia and BVD on NAD(P)H oxidase activities
by the lucigenin method.

The signaling mechanisms underlying the rapid activation of non-phagocytic NAD(P)H
oxidase in vascular cells have been relatively well established. One of the most important
physiological regulators is Ang II [28]. Ang II rapidly activates phospholipase C to increase
intracellular calcium and diacylglycerol levels, which causes the activation of protein kinase C
(PKC). Activated PKC phosphorylates p47phox and induces the release of superoxide from
NOX components. We and other investigators have also reported that high glucose levels
stimulate superoxide production from vascular endothelial cells and smooth muscle cells via a
PKC-dependent activation of NAD(P)H oxidase [12] [13] [14] [15]. In the diabetic state, high
glucose levels and activation of local renin-angiotensin systems may induce the rapid activation
of NAD(P)H oxidase in renal and vascular tissues of diabetic patients and animals [29] [30]. A
previous report showed that the hydrophobic tetrapyrrole structure of bilirubin can inhibit
NAD(P)H oxidase activity directly in a cell-free system [10], probably via inhibition of the
interactions between regulatory components and NOX family members. In the present study, we
showed that bilirubin and biliverdin inhibited NAD(P)H activities in cultured renal mesangial
cells, and inhibited high glucose-induced and Ang II- induced ROS production. The inhibitory
effect of bilirubin and biliverdin on the expression of NAD(P)H oxidase components were also
confirmed. All these findings suggest that bilirubin and biliverdin may inhibit oxidative stress in
diabetic kidneys by their inhibitory effects on both the activation and the overexpression of 
NAD(P)H oxidase. Furthermore, the present study revealed that bilirubin and biliverdin were 
more effective in preventing Ang II-induced expression of NAD(P)H oxidase, TGFβ and 
fibronectin in cultured mesangial cells than maximal doses of other antioxidants such as NAC 
and α-LA, supporting the effectiveness of bilirubin and biliverdin on diabetic nephropathy. The 
detailed molecular mechanisms for the beneficial effects of bilirubin and biliverdin should be 
clarified in future studies.

Accumulating evidence has shown that subjects with higher BIL levels have a lower incidence 
of cardiovascular disease and strokes [31] [32] [33]. Recently, we showed a markedly lower 
prevalence of nephropathy as well as cardiovascular disease in diabetic patients with Gilbert 
syndrome[16]. One report has also shown that serum BIL level is associated with 
microalbuminuria in patients with type 2 diabetes [34]. These epidemiological findings showing 
the relationship between serum BIL level and prevalence of diabetic nephropathy are consistent 
with the present findings implicating that hyperbilirubinemia and BVD administration protect 
against diabetic nephropathy.

In conclusion, we showed for the first time that hyperbilirubinemia and biliverdin 
administration protected against diabetic nephropathy via inhibition of oxidative stress, at least in 
part by down-regulating renal NAD(P)H oxidase, in animal models of diabetes. The present 
findings may lead to novel antioxidant therapies for early stages of diabetic nephropathy.
METHODS

Animals

Male homologous Gunn j/j rats and age-matched heterozygous Gunn rats j/+ were purchased from Clea Japan Inc., Tokyo, Japan. At 8 weeks of age, half of the Gunn j/j rats (n=10) and half of the Gunn j/+ rats (n=10) were injected intraperitoneally with streptozotocin (Sigma, St. Louis, MO) at 80 mg/kg body weight. One or 2 days after injection, the development of diabetes was verified by the presence of hyperglycemia (plasma glucose levels>16.7 mmol). Male C57BL/KsJ db/db mice and their age-matched lean littermates, db/+ mice, were purchased from Clea Japan Inc.. All mice were bred under pathogen-free conditions at Kyushu University Animal Center, Fukuoka, Japan. The animals had free access to tap water and standard chow (Clea Japan Inc.). At 12 weeks of age, half of the db/db (n=8) and half of the db/+ (n=8) mice were randomly chosen to receive powder diet (Clea Japan Inc.) supplemented with biliverdin (5mg/Kg) (Frontier Scientific Inc., Logan, UT) and the rest of the mice were received powder diet without any supplementation, for 2 or 12 weeks. Powder diet was stored at 4°C and kept away from excessive light. All protocols were reviewed and approved by the Committee on the Ethics of Animal Experiments, Graduate School of Medical Science, Kyushu University.

Human mesangium cells and culture

NHMC were obtained from Lonza (Walkersville, MD) and cultured in BulletKit containing 5% FBS, 50mg/ml gentamicin, and 50µg/ml amphotericin-B in a humidified air/5% CO2 atmosphere at 37°C. All cells were studied at passage 4. For more details, see online supplement.

Blood and urine analysis
Plasma concentrations of total and direct BIL were measured, and a 24-h urine sample was collected using metabolic cages to analyze urinary albumin and oxidative stress makers. The well-mixed urine was centrifuged at $7500 \times g$ for 5 min, purged of air with a steam of nitrogen to prevent artificial formation of oxidative stress products, and then stored at $-80^\circ C$ until analysis. Relating to these analyses, see online supplement for details.

**Immunohistochemistry**

Immunostaining for 8-OHdG and NOX4 in the kidney was performed as previously described [24]. Methods are described in the online appendix section.

**Dihydroethidium stain in the renal tissues**

To detect ROS in the renal glomeruli and tubules, DHE (Invitrogen, Carlsbad, CA) stain was performed as previously described [35] with a slightly modified procedure. The detailed method is summarized in the online appendix section.

**Isolation of glomeruli**

Isolation of mice glomeruli was performed using Dynabeads M-450 tosylactivated and Magnetic Particle Concentrator (Dynal A.S., Oslo, Norway) as previously described [36]. We performed a slightly modified procedure. The detailed method is summarized in the online appendix section.

**RNA extraction and quantitative RT-PCR**

Methods are described in the online appendix section.

**Morphologic study**

The mesangial matrix was determined by the presence of PAS-positive and nuclei-free areas.
The detailed method is summarized in the online appendix section.

**Western Blot Analysis**

Methods are described in the online appendix section.

**Measurement of NAD(P)H oxidase activities**

NAD(P)H oxidase activities in renal cortex tissues and mesangial cell homogenates were evaluated by the lucigenin-enhanced chemiluminescence method. Intracellular oxidative stress was evaluated by the DCF-DA staining method. Both detailed methods are summarized in the online appendix section.

**Statistical analysis**

All data are expressed as means ± SEM. Statistical analysis was performed with Student’s t test or one-way ANOVA with Fisher’s PLSD test. P < 0.05 was considered statistically significant.

**Disclosure**

All the authors declared no competing interests.
REFERENCES


ACKNOWLEDGMENTS

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Fig. 2

(a) Urinary 8-OHdG excretion

(b) Urinary 8-epi-PGF$_{2\alpha}$ excretion

Contr DM Contr DM

8weeks 24weeks

µg/g Cre
Fig. 2

**c**

Relative glomerular 8-OHdG intensity (% of control)

**d**

Relative renal tubule 8-OHdG intensity (% of control)

**e**

**f**
Fig. 3

(A) Immunohistochemical analysis of NOX4 expression in the kidney of juvenile mice. (B) Western blot analysis of NOX4 and β-actin expression in the kidney of juvenile mice. **NOX4 expression was significantly increased in the kidneys of j/+ DM mice compared to j/+ and j/j DM mice.**

**NOX4 (67kD)**

**β-actin**
Fig. 3

- **d**: NOX4 mRNA (% of control)
- **e**: p22phox mRNA (% of control)
- **f**: p47phox mRNA (% of control)

Bar charts show gene expression levels for different genotypes (j/j, j/+, j/j) under control (Contr) and DM conditions. Bars are labeled with significance levels: *p < 0.05; **p < 0.01.
Fig. 4

(a) Images showing renal sections from different genotypes: j/+ (top left), j/j (top right), j/+ DM (bottom left), and j/j DM (bottom right).

(b) Graph depicting the relative mesangial area (% of control) for genotypes j/+ (Contr), j/j, j/+ DM, and j/j DM. The bars indicate significant differences with *** indicating p < 0.001.
Fig. 5

mg/g Cre

Ur Alb / Cr Ratio

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Significance levels: *** p < 0.001, ** p < 0.01, * p < 0.05
Fig. 6

(a) Urinary 8-OHdG excretion (µg/g Cre) at 2 weeks and 12 weeks for db/+ BVD, db/db, db/db BVD.

(b) Urinary 8-epi-PGF2α excretion (µg/g Cre) at 2 weeks and 12 weeks for db/+ BVD, db/db, db/db BVD.
Fig. 6

- **Relative glomerular 8-OHdG intensity (% of control)**
  - **e**: Comparison of relative glomerular 8-OHdG intensity for different genotypes and treatments.
  - **f**: Comparison of relative renal tubule 8-OHdG intensity for different genotypes and treatments.

**Genotypes and Treatments**:
- **db/+**: Normal genotype without treatment.
- **db/db**: Obese genotype without treatment.
- **db/+ BVD**: Normal genotype treated with BVD.
- **db/db BVD**: Obese genotype treated with BVD.
Fig. 6

**g**

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**i**

Relative glomerular DHE oxidation intensity (% of control)

![Graph](graph_glomerular.png)

**j**

Relative renal tubule DHE oxidation intensity (% of control)

![Graph](graph_tubule.png)
### Table: NADPH-dependent Superoxide Generation (RLU/min/mg protein)

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**Fig. 6**

![Graph showing NADPH-dependent Superoxide generation](image)
**Fig. 7**

(a) Immunohistochemical staining of NOX4 in db/+ and db/db kidneys.(db+/+ BVD) (db/db BVD)

(b) Hematoxylin and eosin staining of kidneys from db/+ and db/db mice. (db+/+ BVD) (db/db BVD)

(c) Western blot analysis showing increased expression of NOX4 in db/+ BVD and db/db BVD compared to db/+. (β-actin) 67kD
Relative mesangial area (% of control)

Fig. 8

- db/+ vs. db/+ BVD
- db/db vs. db/db BVD
Fig. 8

**g**

![Bar chart showing TGF-β1 mRNA levels.](image)

**h**

![Bar chart showing fibronectin mRNA levels.](image)

TGF-β1 mRNA (% of control)

fibronectin mRNA (% of control)
Lucigenin
NADPH
DPI
Bilirubin
Biliverdin

Fig. 9

Lucigenin
NADPH
DPI
Bilirubin

---

* *= * *= * =

** *= ** ** ** **

Lucigenin
NADPH
DPI
Biliverdin

---

* *= * *= * =

** *= ** ** ** **
Fig. 9

Control

Ang II

Ang II + Bilirubin

Ang II + DPI

Ang II (100nM)

Bilirubin

DPI

(% of control)
Fig. 9

- Glucose: 100 mg/dl, 450 mg/dl
- Bilirubin: 0, 10 µM

Graph showing the effect on (percent of control) with significant differences indicated by **.
**Fig. 10 b**

**Western Blot**

**NOX4**

**β-actin**

67kD

**Graph**

NOX4 (% of control)

- **Angiotensin II**: +, -, +, +, +, +, +
- **Biliverdin**: -, -, +, -, -, -, -
- **Bilirubin**: -, -, -, +, -, -, -
- **NAC**: -, -, -, -, +, -
- **α-LA**: -, -, -, -, -, +
Fig. 10 c
Fig. 10

The bar chart shows the effect of Ang II (1X10^{-7}M) on Fibronectin mRNA expression (% of control) in the presence of various additional agents:
- Ang II (1X10^{-7}M) + Bilirubin (1X10^{-6}M)
- Ang II (1X10^{-7}M) + Biliverdin (1X10^{-6}M)
- Ang II (1X10^{-7}M) + NAC (5X10^{-3}M)
- Ang II (1X10^{-7}M) + α-Lipoic acid (1X10^{-4}M)

Statistical significance is indicated by:
- * for p < 0.05
- ** for p < 0.01
- *** for p < 0.001
Table I  Body weights, blood glucose and serum total / direct bilirubin levels in Gunn j/+ and j/j rats at baseline, 8 weeks and 24 weeks after STZ-injection.

<table>
<thead>
<tr>
<th></th>
<th>j / +</th>
<th>j / +</th>
<th>j / j</th>
<th>j / j</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contr (n=10)</td>
<td>DM (n=10)</td>
<td>Contr (n=10)</td>
<td>DM (n=10)</td>
</tr>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0w</td>
<td>264.0±3.6</td>
<td>262.5±7.4</td>
<td>248.0±3.5</td>
<td>230.0±9.8</td>
</tr>
<tr>
<td>8w</td>
<td>439.0±3.6</td>
<td>278.8±11.1 #</td>
<td>400.0±5.9</td>
<td>219.3±16.6 ##</td>
</tr>
<tr>
<td>24w</td>
<td>548.5±11.0</td>
<td>287.0±9.7 #</td>
<td>444.4±6.8</td>
<td>225.0±19.4 ##</td>
</tr>
<tr>
<td>Blood glucose level (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0w</td>
<td>123.6±2.1</td>
<td>123.9±6.9</td>
<td>123.1±3.7</td>
<td>117.8±9.6</td>
</tr>
<tr>
<td>8w</td>
<td>107.9±3.3</td>
<td>550.9±28.4 #</td>
<td>113.0±1.0</td>
<td>518.3±15.8 ##</td>
</tr>
<tr>
<td>24w</td>
<td>188.9±12.2</td>
<td>556.3±11.1 #</td>
<td>178.9±9.5</td>
<td>524.4±20.8 ##</td>
</tr>
<tr>
<td>Serum total bilirubin level (mg/dl)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8w</td>
<td>0.18±0.04</td>
<td>0.15±0.02</td>
<td>9.47±0.04</td>
<td>7.01±0.43 ##</td>
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<tr>
<td>Serum direct bilirubin level (mg/dl)</td>
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<tr>
<td>8w</td>
<td>0.06±0.01</td>
<td>0.07±0.03</td>
<td>0.64±0.03</td>
<td>0.51±0.03</td>
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</tbody>
</table>

Data are means±SE.  # <0.001 vs j / + Contr;  ## < 0.001 vs. j / j Contr
Table 2  Body weights, blood glucose and serum total / direct bilirubin levels in db/+ and db/db mice at baseline, at 2 weeks and 12 weeks after treatment.

<table>
<thead>
<tr>
<th></th>
<th>db/+ (n=8)</th>
<th>db/db (n=8)</th>
<th>db/+ + BVD (n=8)</th>
<th>db/db + BVD (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0w</td>
<td>28.4±0.4</td>
<td>50.2±0.5 #</td>
<td>29.9±0.7</td>
<td>51.4±0.6 ###</td>
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<tr>
<td>2w</td>
<td>29.7±0.4</td>
<td>51.5±0.5 #</td>
<td>29.8±0.5</td>
<td>51.6±0.4 ##</td>
</tr>
<tr>
<td>12w</td>
<td>32.1±0.7</td>
<td>53.9±1.1 #</td>
<td>33.0±0.7</td>
<td>52.9±0.6 ##</td>
</tr>
<tr>
<td><strong>Blood glucose level (mg/dl)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0w</td>
<td>124.6±4.8</td>
<td>474.6±20.5 #</td>
<td>131.3±6.1</td>
<td>483.0±11.2 ##</td>
</tr>
<tr>
<td>2w</td>
<td>145.8±3.5</td>
<td>545.9±16.6 #</td>
<td>121.3±8.4</td>
<td>530.1±22.7 ##</td>
</tr>
<tr>
<td>12w</td>
<td>154.2±4.4</td>
<td>574.6±13.2 #</td>
<td>145.8±3.5</td>
<td>522.4±43.5 ##</td>
</tr>
<tr>
<td><strong>Serum total bilirubin level (mg/dl)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0w</td>
<td>0.74±0.08</td>
<td>0.70±0.05</td>
<td>0.74±0.07</td>
<td>0.76±0.06</td>
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<tr>
<td>2w</td>
<td>0.98±0.03</td>
<td>0.99±0.04</td>
<td>0.95±0.03</td>
<td>0.98±0.03</td>
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<tr>
<td><strong>Serum direct bilirubin level (mg/dl)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0w</td>
<td>0.26±0.05</td>
<td>0.24±0.03</td>
<td>0.25±0.02</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>2w</td>
<td>0.22±0.02</td>
<td>0.20±0.02</td>
<td>0.21±0.03</td>
<td>0.21±0.02</td>
</tr>
</tbody>
</table>

Data are means±SE. # < 0.01 vs. non-treated db /+, ## < 0.01 vs. treated db /+
FIGURE LEGENDS

Fig. 1

Ur Alb / Cr Ratio in homozygous j/j (n=10) and heterozygous j/+ Gunn rats (n=10) at 8 and 24 weeks after the onset of diabetes. A 24-h urine sample was collected using metabolic cages. The well-mixed urine was centrifuged at 7500×g for 5 min, and then stored at −80°C until analysis. Ur Alb / Cr Ratio (mg/g creatinine) was measured as described in the Methods. DM, streptozotocin-induced diabetic. Contr, non-diabetic. Results are expressed as means±SE. *P<0.05; **P<0.01; *** P<0.001.

Fig.2

Urinary oxidative stress markers in homozygous j/j (n=10) and heterozygous j/+ (n=10) Gunn rats at 8 or 24 weeks after the onset of diabetes. Urinary 8-OHdG excretion (μg/g creatinine) (a), and urinary 8-epi-PGF\(_{2α}\) excretion (μg/g creatinine) (b) were measured as described in the Methods. Results are expressed as means±SE. *P<0.05; **P<0.01.

Immunostaining analysis of renal 8-OHdG. Renal 8-OHdG contents were evaluated by immunostaining analysis. The pictures are representative of the samples of glomeruli (c) and tubules (d) from non-diabetic heterozygous Gunn j/+ rats (n=10), diabetic heterozygous Gunn j/+ rats (n=10), non-diabetic homozygous Gunn j/j rats (n=10) and diabetic homozygous Gunn j/j rats (n=10). The immunostaining intensities of 8-OHdG in the renal glomeruli (e) and tubules (f) were semi-quantitatively evaluated using Scion imaging software (Scion, Frederick, MD). Results are expressed as the mean percentages of the levels in non-diabetic heterozygous Gunn
rats j/+ ±SE. *** P<0.001.

**Fig. 3**

Immunostaining analysis of NOX4 protein levels in renal tissues. The pictures are representative of the samples of glomeruli (a) and tubules (b). Similar results were obtained in all samples from the kidneys of each group (n=10).

NOX4 protein levels were evaluated by Western blot analysis. Pictures are representative of Western blots for NOX4 in renal tissues (c). NOX4 levels were normalized to the level of β-actin, and the results are expressed as the mean percentages of the levels in control heterozygous Gunn j/+ rats ±SE (for all groups, n=10). *** P<0.001.

The levels of mRNA for NOX4, p22phox and p47phox in renal tissues. Total RNA was extracted from the kidneys of each group (n=10) of rats at 24 weeks after the onset of diabetes. NOX4, p22phox and p47phox mRNA levels were measured by real-time RT-PCR (d, e, f). The levels of mRNA were normalized to the levels of β-actin, and the results are expressed as the mean percentages of the levels in control heterozygous Gunn j/+ rats ±SE. *P<0.05; **P<0.01.

**Fig. 4**

Mesangial expansion in homozygous j/j and heterozygous j/+ Gunn rats (a,b). Twenty-four weeks after the onset of diabetes, renal sections were stained with PAS. The pictures are representative of samples from non-diabetic heterozygous Gunn rats j/+ (n=10), non-diabetic homozygous Gunn rats j/j (n=10), diabetic heterozygous Gunn rats j/+ (n=10) and diabetic
homozygous Gunn rats j/j (n=10). Semi-quantitative analysis of the mesangial area. Results are expressed as the mean percentages of the areas in non-diabetic heterozygous Gunn rats j/+ ±SE. ***P< 0.001.

TGF-β1 and fibronectin mRNA and protein levels (c, d, e, f). Total RNA and protein were extracted from the kidneys of each group of rats 24 weeks after the onset of diabetes (For all groups, n=10). TGF-β1 and fibronectin mRNA levels were measured by real-time RT-PCR (c, d). The levels of mRNA were normalized to the levels of β-actin, and the results are expressed as the mean percentages of the levels in control heterozygous Gunn rats j/+ ±SE. TGF-β1 and fibronectin protein levels were evaluated by Western blot analysis (e, f). Pictures are representative of Western blots of TGF-β1 and fibronectin in the renal tissues. The levels of protein were normalized to the levels of β-actin, and the results are expressed as the mean percentages of the levels in control heterozygous Gunn rats j/+ ±SE. *P<0.05; **P<0.01; *** P<0.001.

**Fig. 5**

Effect of biliverdin treatment on Ur Alb / Cr Ratio in db/+ mice (n=8) and db/db mice (n=8). Biliverdin (5 mg/kg) was administered to db/db and db/+ mice starting at 12 weeks of age for 2 or 12 weeks. A 24-h urine sample was collected using metabolic cages. The well-mixed urine was centrifuged at 7500×g for 5 min, and then stored at −80°C until analysis. BVD, biliverdin treated mice. Ur Alb / Cr Ratio (mg/g creatinine) was measured as described in the Methods. *P<0.05, **P<0.01, *** P<0.001.
The effect of biliverdin treatment on urinary 8-OHdG excretion and urinary 8-epi-PGF$_{2\alpha}$ excretion. Urinary 8-OHdG excretion (μg/g creatinine) (a), and urinary 8-epi-PGF$_{2\alpha}$ excretion (μg/g creatinine) (b) were measured after 2 and 12 weeks treatment, as described in the Methods. Results are expressed as means±SE. (For all groups, n=8) *P<0.05, **P<0.01, *** P<0.001.

Immunostaining analysis of renal 8-OHdG. The pictures are representative of the samples of glomeruli (c) and tubules (d) from non-treated db/+ mice (n=8), non-treated db/db mice (n=8), BVD-treated db/+ mice (n=8) and BVD- treated db/db mice (n=8). The immunostaining intensities of 8-OHdG in the renal glomeruli and tubules were semi-quantitatively evaluated using Scion imaging software (Scion, Frederick, MD) (e, f). Results are expressed as the mean percentages of the levels in non-treated db/+ mice ±SE.

*P<0.05; *** P<0.001.

The effect of biliverdin treatment on ROS production was detected by DHE stain in the renal tissues. The pictures are representative of the samples of glomeruli (g) and tubules (h) from non-treated db/+ mice (n=8), non-treated db/db mice (n=8), BVD-treated db/+ mice (n=8) and BVD- treated db/db mice (n=8). The emission intensities of DHE oxidation in the renal glomeruli and tubules were semi-quantitatively evaluated using Scion imaging software (Scion, Frederick, MD) (i, j). Results are expressed as the mean percentages of the levels in non-treated db/+ mice ±SE. *** P<0.001.

NADPH oxidase activity assay for crude cortex homogenates from non-treated db/+ mice (n=8), non-treated db/db mice (n=8), BVD-treated db/+ mice (n=8) and BVD- treated db/db mice (n=8)
(k). After establishing a background rate for 5 min, NADPH was added and the increase in
lucigenin enhanced chemiluminescence was monitored by Flex Station 3. Results are expressed as
means±SE. *P<0.05, **P<0.01. vs. db/db mice.

**Fig. 7**

The effect of biliverdin treatment on NOX4 protein levels in renal tissues. Immunostaining
analysis of NOX4 protein levels in renal tissues. The pictures are representative of the samples
of glomeruli and tubules (a, b). Similar results were obtained in all samples from the kidneys of
each group. (For all groups, n=8)

NOX4 protein levels were evaluated by Western blot analysis (c). The levels of protein were
normalized to the level of β-actin, and the results are expressed as the mean percentages of the
levels in non-treated db/+ mice ±SE. (For all groups, n=8) **P<0.01.

The expression of mRNA levels for the NOX4, p22phox and p47phox in renal tissues. Total
RNA was extracted from the kidneys after 12 weeks biliverdin treatment, and the levels of
mRNA were measured by real-time RT-PCR (d, e, f). The levels of mRNA were normalized to
the levels of β-actin, and the results are expressed as the mean percentages of the levels in
non-treated db/+ mice ±SE. (For all groups, n=8)

*P<0.05; *** P<0.001.

The expression of mRNA levels for NOX4, p22phox and p47phox in the glomeruli. Total RNA
was extracted from the glomeruli after 12 weeks of biliverdin treatment. The each extracted
mRNA from the glomeruli were measured by real-time RT-PCR(g, h, i) The levels of mRNA
were normalized to the levels of β-actin, and the results are expressed as the mean percentages of
the levels in non-treated db/+ mice ±SE. (For all groups, n=8) ** P<0.01.

**Fig.8**

The effect of biliverdin treatment on mesangial expansion (a, b). After 12 weeks treatment, renal
sections were stained with PAS. The pictures are representative of samples from non-treated
db/+ mice (n=8), non-treated db/db mice (n=8), biliverdin (BVD)-treated db/+ mice (n=8) and
BVD-treated db/db mice (n=8). Semi-quantitative analysis of the mesangial area. Results are
expressed as the mean percentages of the areas in non-treated db/+ mice±SE. ***P<0.001.

TGF-β1 and fibronectin mRNA and protein levels (c, d, e, f). Total RNA and protein were
extracted from the kidneys of each group of mice after 12 weeks BVD treatment (For all groups,
n=8). TGF-β1 and fibronectin mRNA levels were measured by real-time RT-PCR (c, d). The
levels of mRNA were normalized to the levels of β-actin, and the results are expressed as the
mean percentages of the levels in non-treated db/+ mice±SE. TGF-β1 and fibronectin protein
levels were evaluated by Western blot analysis (e, f). Pictures are representative of Western blots
of TGF-β1 and fibronectin in the renal tissues. The levels of protein were normalized to the
levels of β-actin, and the results are expressed as the mean percentages of the levels in
non-treated db/+ mice ±SE. *P<0.05; **P<0.01; *** P<0.001.

The expression of mRNA levels for the TGF-β1 and fibronectin in the glomeruli. The extracted
mRNA from the glomeruli were measured by real-time RT-PCR (g, h). The levels of mRNA
were normalized to the levels of β-actin, and the results are expressed as the mean percentages of
the levels in non-treated db/+ mice±SE. *P<0.05; **P<0.01.

Fig. 9

The effect of bilirubin(a) and biliverdin(b) on NAD(P)H oxidase activities in cultured human renal mesangial cells. BIL or BVD were incubated each concentrations for 48 hours as indicated. After establishing a background rate for 5 min, NADPH was added and the increase in lucigenin-enhanced chemiluminescence was monitored by Flex Station 3. Results are expressed as means±SE from 4 independent experiments. *P<0.05, **P<0.01. vs. NADPH.

The effect of bilirubin on increased reactive oxygen species production by Ang II (c) and high glucose (d) stimulation in cultured human endothelial cells as evaluated by DCF-DA staining. The obtained fluorescence images were converted to gray scale images and quantitatively analyzed using NIH image software. Results are expressed as the mean percentages of the levels in control ± SE from 4 independent experiments.

*P<0.05; **P<0.01.

Fig.10

The effect of bilirubin and biliverdin on Ang II (100nmol/l-stimulated expression of NOX4(a), TGF-β1(c) and fibronectin (d) mRNA levels in cultured human mesangium cells. The extracted mRNA were measured by real-time RT-PCR, and levels of each mRNA were normalized to the levels of β-actin. Results are expressed as the mean percentages of the levels in control ± SE .

*P<0.05; **P<0.01; *** P<0.001. vs. control
NOX4 protein levels were also evaluated by Western blot analysis (b). The levels of protein were normalized to the level of β-actin, and the results are expressed as the mean percentages of the levels in control ± SE. *P<0.05. vs. Angiotensin II
RESEARCH DESIGN AND METHODS

Human mesangium cells and culture
Near-confluent NHMC were incubated with BulletKit for 24 h to arrest and synchronize cell growth. After this period, the medium was changed to fresh BulletKit (5% FBS) containing either 100nmol/l Ang II. Cells were also incubated with 100nmol/l Ang II medium in the presence of 1μmol/l bilirubin (Sigma, St Louis, MO), 1μmol/l biliverdin, 5mmol/l NAC (Sigma, St Louis, MO), 100μmol/lα-LA (Sigma, St Louis, MO), for 6, 12, 18 and 24 hours.

Blood and urine analysis
Plasma concentrations of total and direct bilirubin were measured using a commercially available kit (Wako, Osaka, Japan). Urinary albumin concentrations were measured using a Rat or Mouse Albumin ELISA Kit (AKRAL-120 or 121; Shibayagi, Gunma, Japan). Urinary 8-OHdG, a major product of oxidative DNA damage, is produced by enzymatic cleavage after 8-hydroxylation of the guanine base of DNA concentrations, were measured using a competitive enzyme-linked immunosorbent assay kit (8OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan), as previously described [4]. Urinary 8-epi- PG F₂α concentrations were measured using a Urinary Isoprostane ELISA Kit (MED.DIA s.r.l., Italy). The results of these urinary studies are expressed as values corrected to the level of urinary creatinine.

Immunohistochemistry
Immunostaining for 8-OHdG and NOX4 in the kidney was performed as previously described [24]. The images, obtained 8-OHdG -positive areas in the renal tissue, were converted to gray scale images using Photoshop software, and the intensities of 8-OHdG -positive areas were quantitatively analyzed using Scion imaging software (Scion, Frederick, MD). To semi-quantify the 8-OHdG-positive areas, sections were coded and read by an observer unaware of the experimental protocol applied. In each animal from the four experimental groups, 30 glomeruli and tubule fields were examined and averaged for morphometric analysis.

Dihydroethidium stain in the renal tissues
To detect ROS in the renal glomeruli and tubule, DHE (Invitrogen, Carlsbad, CA) stain was performed. The mice were anesthetized under isoflurane, then three hundred microliters of DHE (stock solution, 100 mg/ml in dimethyl sulfoxide at 4 ℃, diluted to 2.5μg /μl with phosphate-buffered saline(PBS) just before use. Then transferred to a 1-ml foiled syringe and DHE solution were administered i.v. through the jugular vein. Ninety minutes after the DHE injection, the mice were anesthetized and killed by transcardial perfusion with 40 ml of 4.0% formaldehyde in PBS. After postfixation the kidneys were sectioned cut into 15 μm thick sections and placed on glass slides. These sections were observed with a fluorescent light microscope (BZ-9000, Keyence, Osaka, Japan). Fluorescence was assessed microscopically at excitation 510–550 nm and emission >580 nm for ethidium bromide detection. The images, obtained DHE oxidation areas, were converted to gray scale images using Photoshop software,
and the intensities of these areas were quantitatively analyzed using Scion imaging software (Scion, Frederick, MD). To semi-quantify the emission intensities of DHE oxidation areas, sections were coded and read by an observer unaware of the experimental protocol applied. In each animal from the four experimental groups, 30 glomeruli and tubule fields were examined and averaged for morphometric analysis.

Isolation of glomeruli

Isolation of mice glomeruli was performed as previously described [36]. Mice were anaesthetized and perfused with 8 x 10^7 Dynabeads M-450 tosylactivated (Dynal A.S., Oslo, Norway) diluted in 40 ml of PBS through the heart. The kidneys were removed, minced into 1 mm^3 pieces and digested in 3mg Collagenase A and 300ug Deoxyribonuclease I(Roche Diagnostics GmbH, Mannheim, Germany) in 3ml HBSS(Invitrogen, Grant Island, NY) at 37°C for 30 min with gentle agitation. The Collagenase-digested tissue was gently pressed through a 100 μm cell strainer (BD Biosciences, Stockholm, Sweden) using a flattened pestle. Then the cell strainer was washed with 10 ml of HBSS. The filtered cells were passed through a new cell strainer without pressing and the cell strainer washed with 10 ml of HBSS. The cell suspension was then centrifuged at 200 x g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 2 ml of HBSS. Finally, glomeruli containing Dynabeads were gathered by a Magnetic Particle Concentrator (Dynal A.S., Oslo, Norway). After aspirate liquid, wash the wall with 300μl HBSS and collect glomeruli. Aspirated liquid was reused and these procedures were performed at least 5 times.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from frozen kidney samples using Isogen (Nippon Gene, Tokyo, Japan), human mesangium cells and mice glomeruli using RNeasy Mini Kit (QIAGEN, Chatsworth,CA) according to the manufacturer’s instructions. Extracted RNA (4 μg) was converted to single-stranded cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The mRNA levels were quantified by quantitative RT-PCR using an iTaq SYBR Green mix (Bio-Rad, Hercules, CA), with the Bio-Rad Chromo 4/Opticon system. The following primer pairs were used:

For rat:

NOX4, 5'- GGGCCTAGGATTGTGTTTGA-3' (sense) and 5'-CTGAGAAATGCAGGCAGTTC-3' (antisense);
p22phox, 5'-TGTGTCAGGAGGTGCTCATCTCTGTCTGCTC-3' (sense) and 5'-AGGACAGCAGCCGGACGTAATTT-3' (antisense);
p47phox, 5'-AGCTCCAGGCAGGTGATG-3' (sense) and 5'-ATCTTTGGCAGGTATGATG-3' (antisense);
TGF-β1, 5'-ATACGCTGAGTGCTGCTGCTC-3' (sense) and 5'-TGGGACTGATCCCATTGATT-3' (antisense);
fibronectin, 5'-GAAAGGCAACCAGCAAGG-3' (sense) and 5'-CTGGAGTCAAGACAGACACA -3' (antisense);
β-actin, 5'-AGCCATGTACGTAGCCATCC -3'(sense) and 5'-CTTCTAGCTGTTGTTGTTG-3' (antisense).

For mouse:
NOX4, 5'-ATTTGGATAGGCTCCAGGCAAAC-3' (sense) and 5'-CACATGGGTTAAGCTTTTGTGAGCA-3' (antisense);
p22phox, 5'-TGGCTACTGCTGGACGTTTCAC-3' (sense) and 5'-CTCCAGCAGACAGATGAC-3' (antisense);
p47phox, 5'-TGGCTACTGCTGGACGTTTCAC-3' (sense) and 5'-CTCCAGCAGACAGATGAC-3' (antisense);
TGF-β1, 5'-GCAGTGAGCGCTGAATCGA -3'(sense) and 5'-GCAGTGAGCGCTGAATCGA -3'(antisense);
fibronectin, 5'-ATCTTGCCAGACATCGA-3'(sense) and 5'-CTCGGAGATCGCAAGCAG-3' (antisense);
TGF-β1, 5'-GCAGTGAGCGCTGAATCGA -3'(sense) and 5'-GCAGTGAGCGCTGAATCGA -3'(antisense);
fibronectin, 5'-ACATGGCTTITAGGCAGACAA -3'(sense) and 5'-ACATGGCTTITAGGCAGACAA -3'(antisense);

For human:
NOX4, 5'- TGTCCCTTTGCTTACAGATT-3' (sense) and 5'-TGAGCTCTGAGGACAAACA-3' (antisense);
TGF-β1, 5'-CTCCAGACCTGAGCAGACATCGA-3' (sense) and 5'-CTCCAGACCTGAGCAGACATCGA-3' (antisense);
fibronectin, 5'-ACCAACCTACGGATGACTCG-3' (sense) and 5'-GGACTTCGAGCAAGAGTGG-3' (antisense);
β-actin, 5'-GGACTTCGAGCAAGAGTGG-3' (sense) and 5'-GGACTTCGAGCAAGAGTGG-3' (antisense).

The linearity of the amplifications as a function of cycle number was tested in preliminary experiments, and each mRNA expression levels were normalized to the expression levels of the housekeeping gene β-actin.

**Morphologic study**

Each renal section were extracted from homozygous j/j and heterozygous j/+ Gunn rats at 24 weeks after onset of diabetes, and db/db mice and db/+ mice at 24 weeks of age (after 12 weeks of treatment). For analysis of the glomeruli, sections were stained with periodic acid-Schiff (PAS). The mesangial matrix was determined by the presence of PAS-positive and nuclei-free areas in the mesangium. The images, obtained PAS-positive and nuclei-free areas, were converted to gray scale images using Photoshop software, and the intensities of these areas were quantitatively analyzed using Scion imaging software (Scion, Frederick, MD). To semi-quantify mesangial expansion, sections were coded and read by an observer unaware of the experimental protocol applied. In each animal from the four experimental groups, 30 glomeruli were examined and averaged for morphometric analysis.
Western Blot Analysis

For total protein extracts and Western blot analysis of NOX4, TGF-β1 and fibronectin, renal tissues were homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA), and centrifuged for 5 minutes at 16,000 rpm. Protein concentrations were determined using a BCATM Protein Assay Kit (Pierce Biotechnology, IL, USA); 100 μg protein/lane was separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (7.5% for NOX4 and TGF-β1 : 5% for fibronectin) and transferred to PVDF membrane (Bio-Rad Laboratories Inc., Hercules, California, USA). After blockade of nonspecific binding sites, membranes were incubated overnight at 4°C with rabbit polyclonal anti-NOX4 (1:1500 ; ab60940; Abcam), rabbit polyclonal anti-TGF-β1 (1:1000 ; ab53169; Abcam,Cambridge, UK) and mouse monoclonal anti-fibronectin (1:2500 ; #610077; BD Bioscience, San Jose, CA), followed by HRP-conjugated sheep anti-mouse IgG antibody (1:5000; Amersham Pharmacia Biosciences, Buckinghamshire, UK) or donkey anti-rabbit IgG antibody (1:5000; Amersham Pharmacia Biosciences, Buckinghamshire, UK) as secondary antibody. We used the ECL Plus system (Amersham Pharmacia Biosciences, Buckinghamshire, UK) for detection.

Measurement of NAD(P)H oxidase activities

Normal human mesangial cells were purchased from Lonza (Walkersville, MD). Mesangial cells were cultured in a Mesangial Cell Growth Medium (Lonza) containing 5% fetal calf serum. The cells from 2nd to 4th passages were used in the experiments. Cellular production of superoxide anion was determined by the lucigenin-enhanced chemiluminescence. For the experiments, after NHMC were incubated with or without various concentrations of bilirubin and biliverdin for 48 h, they were detached with trypsin/EDTA and were homogenized in modified HEPES buffer containing (mmol/L) NaCl 140, KCl 5, MgCl2 0.8, CaCl2 1.8, Na2HPO4 1, HEPES 25, and 1% glucose (pH 7.2). Renal cortex tissues from mice were also homogenized in buffer above mentioned, and centrifuged for 15 minutes at 16,000 rpm. Immediately before recording, NADPH (100 μM) and dark-adapted lucigenin (10 μM) were added to cells or tissues homogenates. Light emission was recorded every 10 seconds for 10 minutes and was expressed as relative light units. Experiments were performed in triplicate. In each experiment, as a positive control, DPI(10 μM) was added before NADPH addition and recording of chemiluminescence. Superoxide production was expressed as relative light units per milligrams of protein. Protein content was measured using a BCATM Protein Assay Kit (Pierce Biotechnology, IL, USA).

Intracellular ROS production was evaluated by DCF-DA (Molecular Probes, Eugene, OR) dye method. For the experiments, the cells were placed into glass-bottomed dishes (MatTek, Ashland, MA). When the cells reached the loose confluent layer, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 1% FBS and 100 mg/dl glucose for 24 hrs. Then, the cells were replaced with test media for the indicated intervals and washed with phenol-red free Hanks, and then loaded with 2 μM DCF-DA, a nonfluorescent compound that freely permeates cells and interacts with intracellular oxidants to form fluorescence compound DCF. After 20 min, digital images of DCF fluorescence were obtained with a fluorescence
microscope system (Olympus, Tokyo, Japan) at an excitation wavelength of 488 nM (argon laser) using a 515 nm long-pass emission filter. The obtained fluorescence images were converted to gray scale images using Photoshop software, and the fluorescence intensities were quantitatively analyzed using the NIH image software.