

# Clinical and experimental evidence for oxidative stress as an exacerbating factor of diabetes mellitus

Ryoichi Takayanagi,<sup>1,\*</sup> Toyoshi Inoguchi<sup>1,2</sup> and Keizo Ohnaka<sup>3</sup>

<sup>1</sup>Department of Internal Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

<sup>2</sup>Innovation Center for Medical Redox Navigation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

<sup>3</sup>Department of Geriatric Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

(Received 9 November, 2010; Accepted 12 November, 2010; Published online 28 December)

The involvement of reactive oxygen species in various diseases has been demonstrated almost *in vitro* or in animal studies and clinical studies supporting the involvement of reactive oxygen species are very few. Bilirubin has been recognized as an important antioxidant and also shown to have an inhibitory effect on the activity of NADPH oxidase, which may be an important source for superoxide production in various tissues. When the prevalence of vascular complications was compared in diabetic patients with and without a congenital hyperbilirubinemia (Gilbert syndrome), the prevalence of retinopathy, macroalbuminuria and coronary artery disease in patients with Gilbert syndrome was about 20% of that in those without Gilbert syndrome. For study of lifestyle-related diseases, the Fukuoka Cohort was constructed from 2003 to 2009 in Kyushu area in Japan, which contains a total of 12,949 persons. Cross-sectional study of the Fukuoka Cohort revealed an inverse relation between serum bilirubin level and the prevalence of type 2 diabetes mellitus. A precursor of bilirubin, biliverdin-treated db/db mice exhibited less albuminuria and nephropathic changes. These effects were paralleled with normalization of oxidative stress markers and expression of NAD(P)H oxidase subunits in kidney. These results suggested that oxidative stress is an exacerbating factor of type 2 diabetes mellitus and that antioxidant therapies are of value to diabetic nephropathy.

**Key Words:** bilirubin, Gilbert syndrome, diabetes, cohort, Gunn rat

It has been shown that reactive oxygen species (ROS) are involved in the development of lifestyle-related diseases such as atherosclerosis, myocardial infarction, cerebrovascular disease, diabetes mellitus, cancer, and even osteoporosis. However, the involvement of oxidative stress in various diseases has been demonstrated almost *in vitro* or in animal studies and direct clinical studies supporting the involvement of ROS are very few. In clinical studies, the participation of ROS has been suggested indirectly, for instance, an antagonist of type 1 angiotensin II receptor (angiotensin receptor blocker: ARB) was shown not only to improve hypertension but also to decrease incidence of diabetes mellitus in large-scale clinical trials. Suppression of diabetes mellitus is thought to be due to repression of insulin resistance by ARB, however, angiotensin II has been suggested to promote ROS production *in vitro*. On the other hand, negative data about the participation of ROS in lifestyle-related diseases were recently reported in a randomized, double-blind, placebo-controlled factorial long-term trial of vitamin E and vitamin C, in which neither vitamin E nor vitamin C supplementation reduced the risk of major cardiovascular events.<sup>(1)</sup> Diabetes mellitus leads to

microvascular complications such as retinopathy, nephropathy and neuropathy, but also is an important independent risk factor for cardiovascular disease. Several well-researched theories have been proposed to explain how chronic hyperglycemia or post-prandial hyperglycemia causes the micro- or macro-vascular derangements. These theories include increased polyol pathway flux, increased advanced glycation end-product (AGE) formation,<sup>(2)</sup> activation of protein kinase C (PKC)<sup>(3)</sup> and increased oxidative stress.<sup>(4)</sup> Recent reports including ours have shown that a PKC-dependent activation of NAD(P)H oxidase (NOX) may be a major source of increased oxidative stress in vascular tissues of diabetes.<sup>(5–10)</sup> Oxidative stress also has been paid increasing attention to as a common underlying mechanism for progressive  $\beta$ -cell dysfunction as well as diabetic vascular complications.<sup>(11)</sup> Bilirubin has been recognized as an important antioxidant<sup>(12)</sup> and also shown to have an inhibitory effect on the activity of NOX.<sup>(13,14)</sup> Higher concentrations of serum bilirubin were related to decreased risk of coronary artery disease<sup>(15–18)</sup> and stroke.<sup>(19)</sup> In this article, we propose the strong involvement of ROS in the development of type 2 diabetes mellitus and its complications by presenting clinical and experimental animal model data about analysis of relationship between hyperbilirubinemia and diabetes mellitus.

## Gilbert Syndrome and Prevalence of Diabetic Vascular Complications

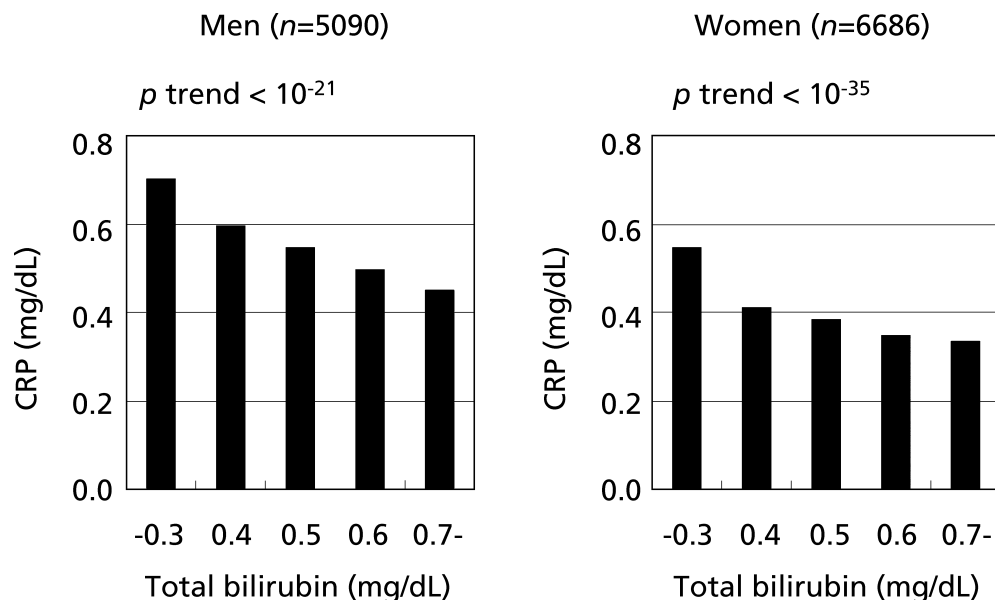
We compared the prevalence of vascular complications in diabetic patients with and without Gilbert syndrome (GS), a congenital hyperbilirubinemia. Screening of 5,080 diabetic patients who visited Kyushu University Hospital and 12 other hospitals and clinics in the Kyushu District of Japan from April to June 2006 yielded 96 consecutive patients with GS, all of whom were enrolled. Determination of GS was based on the presence of unconjugated bilirubin-dominant hyperbilirubinemia (serum bilirubin level >1.2 mg/dl) for 3 or more months, in the absence of hemolytic disease and/or hepatic dysfunction. Patients with diabetes for less than 5 years were excluded. For vascular outcomes, retinopathy was assessed by funduscopic examination; macroalbuminuria was defined as urinary albumin to creatinine ratio of >300 mg/g; coronary artery disease was defined as a history of acute myocardial infarction, angina confirmed by clinically significant obstruction on coronary angiography, or revasculariza-

\*To whom correspondence should be addressed.  
E-mail: takayana@intmed3.med.kyushu-u.ac.jp

**Table 1.** Adjusted odds ratio for retinopathy, macroalbuminuria, coronary artery disease and cerebrovascular disease in diabetic patients with Gilbert syndrome compared with those without it.

Vascular complications	Odds ratio	95% C.I.	$\chi^2$ value	<i>p</i> value
Retinopathy	0.216	0.104–0.446	17.06	<0.0001
Macroalbuminuria	0.205	0.061–0.687	6.60	0.0102
Coronary artery disease	0.206	0.048–0.885	4.51	0.0336
Cerebrovascular disease	0.502	0.200–1.843	0.77	0.2172

In stepwise logistic regression analysis, the prevalence of retinopathy was significantly associated with Gilbert syndrome (GS), duration of diabetes, HbA<sub>1c</sub>; the prevalence of macroalbuminuria was associated with GS, duration of diabetes, triglyceride; the prevalence of coronary artery disease was associated with GS, duration of diabetes, systolic blood pressure; the prevalence of cerebrovascular disease was associated with age, triglyceride, but not GS. Odds ratio for retinopathy, macroalbuminuria, coronary artery disease and cerebrovascular disease was adjusted for significantly determinant variables, respectively. Reproduced from (20) with permission.



**Fig. 1.** Geometric means of serum high sensitivity C-reactive protein according to serum total bilirubin concentrations in men and women. Adjusted for age (continuous variable), smoking (never, past, and current with a consumption of <20 or ≥20 cigarettes per day), alcohol use (never, former and current with a consumption of <30, 30–59 or 60 ml of alcohol per day), body mass index (<22.5, 22.5–24.9, 25.0–27.4, and ≥27.5 kg/m<sup>2</sup>), job-related and leisure-time physical activity (each categorized at quartiles), serum alanine aminotransferase (<40, 40–80, and >80 U/L), and serum gamma-glutamyltransferase (categorized at sex-specific quartiles). Reproduced from (22) with permission.

tion; cerebrovascular disease was defined as a history of symptomatic stroke, confirmed by brain imaging. Metabolic variables and the prevalence of vascular complications were compared to those of 426 diabetic patients without GS who visited Kyushu University Hospital during the same period. Serum bilirubin levels were approximately 2.5-fold higher in patients with GS. The adjusted odds ratio for the association of GS with retinopathy, macroalbuminuria or coronary artery disease was 0.20–0.22 (Table 1), suggesting that sustained hyperbilirubinemia can reduce diabetic complications by almost 80% by its antioxidant action and an inhibitory effect on NOX.<sup>(20)</sup>

### Serum Bilirubin Level and Type 2 Diabetes Mellitus in Middle-Aged and Elderly Japanese Men and Women

**Bilirubin, high sensitivity C-reactive protein (hs-CRP) and glycated hemoglobin.** The study subjects were participants in the baseline survey of the Kyushu University Fukuoka Cohort Study. Residents aged 49 to 76 years in the East Ward of Fukuoka City were invited to participate in the study by mail. During the period between February 2004 and August 2007, a total of 12,949 persons participated in the survey. A total of 531

subjects were excluded for the following reasons: current medical care for chronic hepatitis, liver cirrhosis or liver cancer (*n* = 233), a prior history of liver cancer (*n* = 3), aspartate aminotransferase (AST) or alanine aminotransferase (ALT) greater than 3-fold of the upper limit of the normal range, i.e., >120 U/L (*n* = 285), total bilirubin >3.0 mg/dl (*n* = 3), serum CRP >10 mg/L (*n* = 212), or serum creatinine >2.0 mg/dl (*n* = 44). Additionally, 12 persons were excluded because of missing values for liver enzymes (*n* = 4), creatinine (*n* = 6), alcohol use (*n* = 4), and leisure-time physical activity (*n* = 1). Of the remaining 12,400 subjects, 624 under medication for diabetes mellitus were excluded in the analysis on hs-CRP and hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), but were included in the analysis on prevalent diabetes. Age-adjusted geometric means of hs-CRP were progressively lower in both men and women with higher values of total bilirubin. *p* values for trends of inverse associations were extremely small without and with adjustment for behavioral factors and liver enzymes (Fig. 1). Bilirubin concentrations also showed highly statistically significant, inverse associations with HbA<sub>1c</sub> concentrations in men and women, regardless of adjustment for the behavioral covariates and liver enzyme activities (Fig. 2). The inverse association of HbA<sub>1c</sub> with total bilirubin was still significant after adjustment

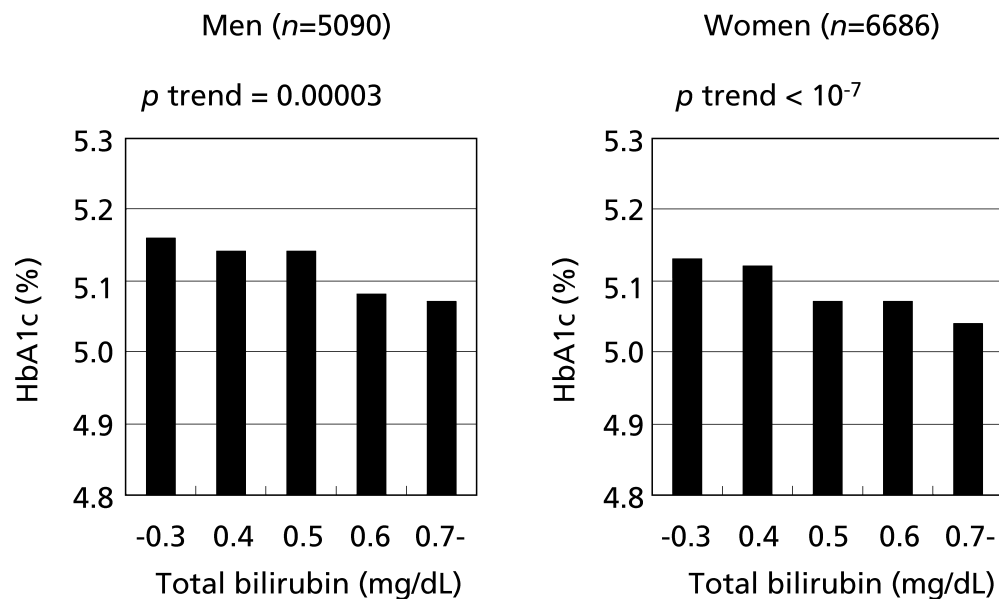


Fig. 2. Geometric means of serum glycosylated hemoglobin according to serum total bilirubin concentrations in men and women. Adjusted for covariates as in the legend for Fig. 1. Reproduced from (22) with permission.

Table 2. Adjusted odds ratios of prevalent diabetes mellitus according to serum total bilirubin.

Total bilirubin concentration (mg/dl)	≤0.3	0.4	0.5	0.6	≥0.7
Number of subjects	2,141	2,897	2,693	2,957	1,712
Number of prevalent cases (%)	171 (8.0)	232 (8.0)	170 (6.3)	216 (7.3)	118 (6.9)
Adjusted OR (95% C.I.)	1.00 (referent)	1.00 (0.81–1.24)	0.73 (0.58–0.91)	0.80 (0.64–1.00)	0.73 (0.56–0.94)

Odds ratios (OR) were adjusted for sex, age, smoking, alcohol use, body mass index, job-related and leisure-time physical activity, alanine aminotransferase, and gamma-glutamyltransferase. *P* for trend based on regression coefficient for a variable with ordinal values assigned to bilirubin categories was 0.002. *p* for trend for OR adjusted only for sex and age was 0.00003 and that for OR adjusted further for C-reactive protein was 0.01. Reproduced from (22) with permission.

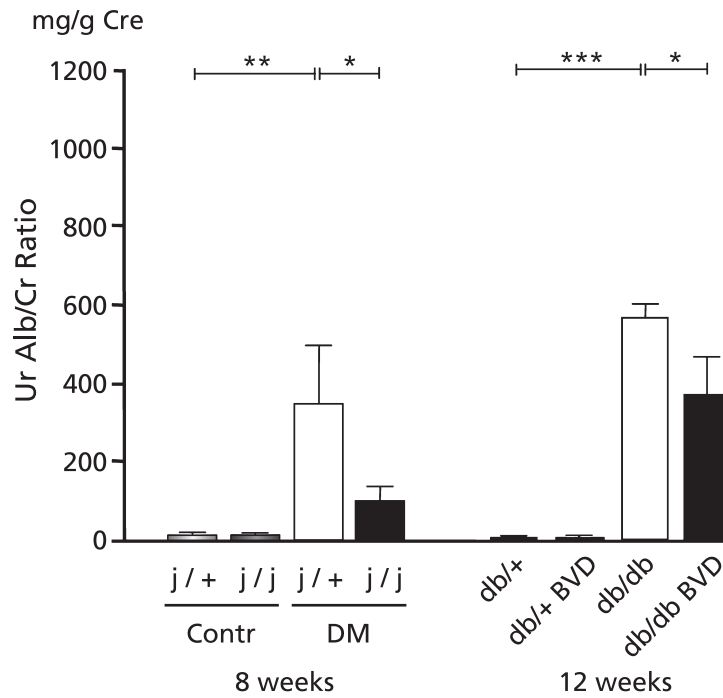
for hs-CRP ( $p = 0.0004$  for men and  $p < 10^{-5}$  for women). Likewise, unconjugated bilirubin concentrations were associated inversely with hs-CRP and HbA<sub>1c</sub>.

**Bilirubin and prevalent diabetes mellitus.** Prevalent cases of type 2 diabetes numbered 907 (602 men and 305 women). The sex- and age-adjusted odds ratios of diabetes decreased almost progressively with increasing concentrations of total bilirubin, showing a statistically highly significant trend. With adjustment for the behavioral factors and liver enzymes, the association was somewhat attenuated. Decreased odds ratios of almost the same magnitude were noted in the three categories of total bilirubin  $\geq 0.5$  mg/dl. Further attenuation of the association occurred with additional adjustment for hs-CRP, but the trend remained statistically significant (Table 2). The analysis was repeated for unconjugated and conjugated bilirubin, and the associations were similar to those observed for total bilirubin. Pancreatic islet is particularly susceptible to damage by oxidative stress because of the lowest levels of intrinsic anti-oxidant defenses.<sup>(21)</sup> These data suggested that higher concentrations of bilirubin may suppress the development of type 2 diabetes through its anti-oxidative effect.<sup>(22)</sup>

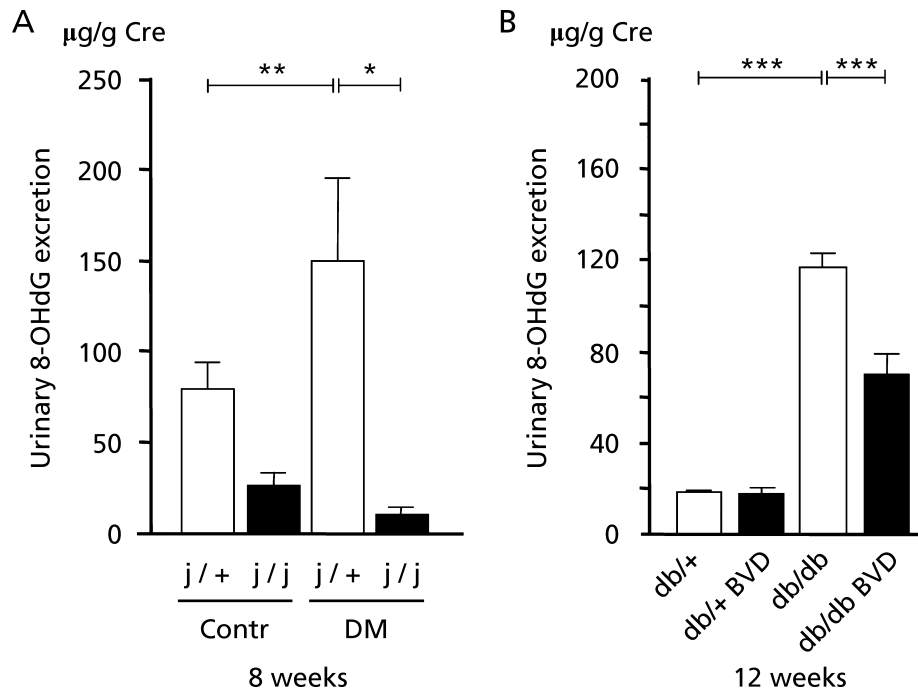
### Hyperbilirubinemic Rats and Diabetic Complications

**Hyperbilirubinemic Gunn rats.** Hereditary hyperbilirubinemic homologous Gunn j/j rats and age-matched control heterozygous Gunn rats j/+ rats were induced to diabetes by intraperitoneal injection of streptozotocin. At 8 weeks after the onset of diabetes,

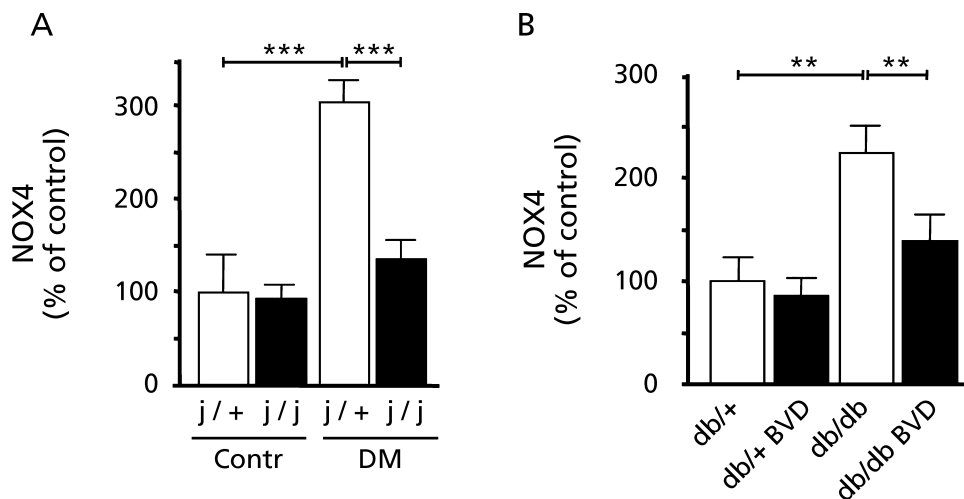
the total serum bilirubin levels were  $0.15 \pm 0.02$  and  $0.18 \pm 0.04$  mg/dl in diabetic and non-diabetic Gunn j/+ rats, respectively, and  $7.01 \pm 0.43$  and  $9.47 \pm 0.04$  mg/dl in diabetic and non-diabetic Gunn j/j rats, respectively. Diabetic Gunn j/+ rats exhibited marked increases in the amounts of urinary albumin excretion compared with non-diabetic Gunn j/+ rats at 8 weeks after the onset of diabetes, whereas diabetic Gunn j/j rats exhibited significantly less urinary albumin excretion than diabetic Gunn j/+ rats (Fig. 3). Urinary excretion levels of a systemic oxidative stress marker, 8-hydroxy-2'-deoxyguanosine (8-OHdG), were significantly higher in diabetic Gunn j/+ rats than in non-diabetic Gunn j/+ rats at 8 weeks. The diabetes-induced increases in 8-OHdG at 8 weeks were completely prevented in diabetic hyperbilirubinemic Gunn j/j rats (Fig. 4A). 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. These increases in 8-OHdG staining intensities in glomeruli and tubules were completely prevented in diabetic Gunn j/j rats. Furthermore, we examined the expression of renal NOX components by 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. 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. All of these changes were completely prevented in diabetic Gunn j/j rats,



**Fig. 3.** Urinary albumin/creatinine (Ur Alb/Cr) ratio (mg/g creatinine) in homozygous *j/j* ( $n = 10$ ) and heterozygous *j/+* Gunn rats ( $n = 10$ ) at 8 weeks after the onset of diabetes, and effect of biliverdin treatment for 12 weeks on Ur Alb/Cr ratio in *dbl/+* mice ( $n = 8$ ) and *dbl/db* mice ( $n = 8$ ). A 24 h urine sample was collected using metabolic cages. DM: streptozotocin-induced diabetic, Contr: non-diabetic, BVD: biliverdin treated mice. Results are expressed as means  $\pm$  SE. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . Reproduced from (23) with permission.



**Fig. 4.** A: Urinary 8-OHdG excretion ( $\mu\text{g/g creatinine}$ ) in homozygous *j/j* ( $n = 10$ ) and heterozygous *j/+* ( $n = 10$ ) Gunn rats at 8 weeks after the onset of diabetes. B: The effect of biliverdin treatment for 12 weeks on urinary 8-OHdG excretion (for all groups,  $n = 8$ ). Results are expressed as means  $\pm$  SE. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . Reproduced from (23) with permission.



**Fig. 5.** Western blot analysis of NOX4 protein levels in renal tissues of Gunn rats A and db mice B. NOX4 levels were normalized to the level of  $\beta$ -actin, and the results are expressed as the mean percentages of the levels in non-treated control rats. Results are expressed as means  $\pm$  SE. \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . Reproduced from (23) with permission.

which showed levels comparable to those in control Gunn  $j/+$  rats (Fig. 5A). 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. The PAS-positive and nuclei-free mesangial area was markedly increased in the glomeruli of diabetic Gunn  $j/+$  rats. Diabetic Gunn  $j/j$  rats showed complete prevention of mesangial expansion.

**Biliverdin-administered db/db mice.** A rodent model of type 2 diabetes, db/db mice, were fed with powder diet supplemented with or without biliverdin (5 mg/kg) at 12 weeks of age. Biliverdin administration orally for 2 weeks and 12 weeks did not significantly affect body weights or blood glucose levels. Oral administration of this dose of biliverdin did not induce a significant elevation in serum bilirubin levels, although an intraperitoneal injection of the same dose induced a slight elevation in serum bilirubin levels at 0.5 h, with rapid return to the basal levels at 6 h after injection. 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 biliverdin administration. Biliverdin administration significantly attenuated such increases in urinary albumin excretion in db/db mice (Fig. 3). Urinary 8-OHdG excretion was significantly higher in db/db mice than in control db/+ mice. Biliverdin administration reduced these markers in db/db mice to control levels (Fig. 4B). The protein levels of NOX4 were also significantly increased in renal tissues of db/db mice. Biliverdin administration normalized all of these changes in db/db mice to the control levels (Fig. 5B). We also confirmed the effect of biliverdin to intracellular production of superoxide in the renal tissues by dihydroethidium stain. The oxidized dihydroethidium signals were significantly higher in db/db mice than those in control mice at 12 weeks. Biliverdin administration completely normalized oxidized dihydroethidium (DHE) signals to the control levels. Mesangial expansion was found in db/db mice at the age of 24 weeks, which was completely prevented by biliverdin administration. The effect of bilirubin and biliverdin on NOX activities in cultured human mesangial cells were evaluated by the lucigenin method. Pretreatment of the cells with both bilirubin and biliverdin for 24 h reduced NOX activities in a dose-dependent manner.

Gunn rats, which exhibit a marked elevation of plasma unconjugated biliverdin 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.<sup>(23)</sup> 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. Administration of biliverdin (5 mg/kg) protected against both albuminuria and renal mesangial expansion in db/db mice. Since serum biliverdin enters cells rapidly and is converted to bilirubin by biliverdin reductase, it is likely that the beneficial effect of biliverdin administration may be due to increased levels of intracellular bilirubin levels generated from exogenously administered biliverdin, rather than increased levels of serum bilirubin or biliverdin.

The present study suggested that the mechanism underlying these beneficial effects of hyperbilirubinemia and biliverdin administration may be the inhibition of oxidative stress, evaluated by systemic oxidative stress markers. The present study revealed that biliverdin administration induced down-regulation of NOX components 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 NOX may be the major sources of increased ROS production in the vascular tissues of diabetic animals and patients, and that high glucose levels stimulate superoxide production from vascular endothelial cells and smooth muscle cells via a PKC-dependent activation of NOX.<sup>(5-7,9)</sup> The non-phagocytic NOX 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.<sup>(24)</sup> The isoform NOX4 was cloned from the kidney, where it was found to be highly expressed.<sup>(25,26)</sup> 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.<sup>(10,27,28)</sup> The present results suggest that down-regulation of NOX components, especially NOX4, by

hyperbilirubinemia and biliverdin administration may play an important role in the inhibition of oxidative stress in the kidneys of diabetic rodents.

In conclusion, we showed using bilirubin as a tool that oxidative stress is an exacerbating factor of type 2 diabetes mellitus and propose that antioxidant therapies are of value to diabetic nephropathy.

## Abbreviations

ROS reactive oxygen species  
ARB angiotensin receptor blocker

AGE advanced glycation end-product  
PKC protein kinase C  
NOX NAD(P)H oxidase  
GS Gilbert syndrome  
AST aspartate aminotransferase  
ALT alanine aminotransferase  
hs-CRP high sensitivity C-reactive protein  
HbA<sub>1c</sub> hemoglobin A<sub>1c</sub>  
8-OHdG 8-hydroxy-2'-deoxyguanosine  
DHE dihydroethidium  
UDPGT-1 uridine diphosphate glucuronosyl transferase-1

## References

- 1 Sesso HD, Buring JE, Christen WG, and *et al.* Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA* 2008; **300**: 2123–2133.
- 2 Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 1988; **318**: 1315–1321.
- 3 Inoguchi T, Battan R, Handler E, Sportsman JR, Health W, King GR. Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci USA* 1992; **89**: 11059–11063.
- 4 Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991; **40**: 405–412.
- 5 Inoguchi T, Li P, Umeda F, and *et al.* High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 2000; **49**: 1939–1945.
- 6 Hink U, Li H, Mollnau H, and *et al.* Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* 2001; **88**: E14–E22.
- 7 Kim YK, Lee MS, Son SM, and *et al.* Vascular NADH oxidase is involved in impaired endothelium-dependent vasodilation in OLETF rats, a model of type 2 diabetes. *Diabetes* 2002; **51**: 522–527.
- 8 Guzik TJ, Mussa S, Gastaldi D, and *et al.* Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* 2002; **105**: 1656–1662.
- 9 Inoguchi T, Sonta T, Tsubouchi H, and *et al.* Protein kinase C-dependent increase in reactive oxygen species (ROS) production in vascular tissues of diabetes: role of vascular NAD(P)H oxidase. *J Am Soc Nephrol* 2003; **14**: S227–S232.
- 10 Sonta T, Inoguchi T, Tsubouchi H, and *et al.* Evidence for contribution of vascular NAD(P)H oxidase to increased oxidative stress in animal models of diabetes and obesity. *Free Radic Biol Med* 2004; **37**: 115–123.
- 11 Tanaka Y, Gleason CE, Tran PO, and *et al.* Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants. *Proc Natl Acad Sci USA* 1999; **96**: 10857–10862.
- 12 Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987; **235**: 1043–1046.
- 13 Kwak JY, Takeshige K, Cheung BS, Minakami S. Bilirubin inhibits the activation of superoxide-producing NADPH oxidase in a neutrophil cell-free system. *Biochim Biophys Acta* 1991; **1076**: 369–373.
- 14 Lanone S, Bloc S, Foresti R, and *et al.* Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxemic shock in rats. *Faseb J* 2005; **19**: 1890–1892.
- 15 Schwertner HA, Jackson WG, Tolan G. Association of low serum concentration of bilirubin with increased risk of coronary artery disease. *Clin Chem* 1994; **40**: 8–23.
- 16 Breimer LH, Wannamethee G, Ebrahim S, Shaper AG. Serum bilirubin and risk of ischemic heart disease in middle-aged British men. *Clin Chem* 1995; **41**: 1504–1508.
- 17 Hopkins PN, Wu LL, Hunt SC, James BC, Vincent GM, Williams RR. Higher serum bilirubin is associated with decreased risk for early familial coronary artery disease. *Arterioscler Thromb Vasc Biol* 1996; **16**: 250–255.
- 18 Djoussé L, Levy D, Cupples LA, Evans JC, D'Agostino RB, Ellison RC. Total serum bilirubin and risk of cardiovascular disease in the Framingham offspring study. *Am J Cardiol* 2001; **87**: 1196–1200.
- 19 Perlstein TS, Pande RL, Creager MA, Weuve J, Beckman JA. Serum total bilirubin level, prevalent stroke, and stroke outcomes: NHANES 1999–2004. *Am J Med* 2008; **121**: 781–788.
- 20 Inoguchi T, Sasaki S, Kobayashi K, Takayanagi R, Yamada T. Relationship between Gilbert syndrome and prevalence of vascular complications in patients with diabetes. *JAMA* 2007; **298**: 1398–1400.
- 21 Robertson RP. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *J Biol Chem* 2004; **279**: 42351–42354.
- 22 Ohnaka K, Kono S, Inoguchi T, and *et al.* Inverse associations of serum bilirubin with high sensitivity C-reactive protein, glycated hemoglobin, and prevalence of type 2 diabetes in middle-aged and elderly Japanese men and women. *Diabetes Res Clin Pract* 2010; **88**: 103–110.
- 23 Fujii M, Inoguchi T, Sasaki S, and *et al.* Bilirubin and biliverdin protect rodents against diabetic nephropathy by downregulating NAD(P)H oxidase. *Kidney Int* 2010; **78**: 905–919.
- 24 Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 2003; **285**: R277–R297.
- 25 Shiose A, Kuroda J, Tsuruya K, and *et al.* A novel superoxide-producing NAD(P)H oxidase in kidney. *J Biol Chem* 2001; **276**: 1417–1423.
- 26 Geiszi M, Kopp JB, Várnai P, Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci USA* 2000; **97**: 8010–8014.
- 27 Etoh T, Inoguchi T, Kakimoto M, and *et al.* Increased expression of NAD(P)H oxidase subunits, NOX4 and p22phox, in the kidney of streptozotocin-induced diabetic rats and its reversibility by interventive insulin treatment. *Diabetologia* 2003; **46**: 1428–1437.
- 28 Fujii M, Inoguchi T, Maeda Y, and *et al.* Pitavastatin ameliorates albuminuria and renal mesangial expansion by downregulating NOX4 in db/db mice. *Kidney Int* 2007; **72**: 473–480.