Hyperbilirubinemia Reduces the Streptozotocin-Induced Pancreatic Damage through Attenuating the Oxidative Stress in the Gunn Rat

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Oxidative stress is an important pathogenic factor in diabetes. Bilirubin may serve a cytoprotective function as an anti-oxidant. The Gunn rat lacks the enzyme uridine-diphosphate glucuronosyltransferase that is responsible for conjugation of bilirubin, exhibiting elevation of plasma bilirubin. We examined the effect of hyperbilirubinemia on the pancreatic damage caused by streptozotocin (STZ) in the Gunn rat. Male Wistar rats and male Gunn rats were treated with STZ (WS and GS groups, respectively) or vehicle (WC and GC groups, respectively). All 5 rats in the WS group developed diabetes, defined as fasting blood glucose 300 mg/dL or more, at 3 days, whereas only 2 of the 5 GS rats became diabetic at 7 days after STZ injection. Without insulin supplement at 7 days after STZ injection, the WS group displayed higher levels of fasting blood glucose (510.3 ± 50.3 vs. 236.4 ± 42.5 mg/dL, p = 0.003) and HbA1c (5.0 ± 0.1 vs. 3.9 ± 0.1, p = 0.001), compared to those of GS group. In Wistar rats, STZ induced apoptosis of the pancreatic islet cells, accompanied with activation of NADPH oxidase and increased production of reactive oxygen species and nitric oxide, but not in Gunn rats. Moreover, in a rat insulinoma cell line (RIN-m5F), pre-treatment with bilirubin (0.1 mg/dL) decreased cell death and apoptosis caused by STZ, and also reduced H2O2 production. Considering the protective effect of hyperbilirubinemia against STZ-induced injury, we postulate that bilirubin could be a potential therapeutic modality for oxidative stress of pancreas islets.

Keywords: bilirubin; streptozotocin; NADPH oxidase; reactive oxygen species; diabetes


Heme is degraded by heme oxygenase, producing equimolar quantities of carbon monoxide, iron, and biliverdin. The latter is reduced to unconjugated bilirubin through biliverdin reductase. Products of heme degradation were once considered to be toxic metabolites, but have been recognized for their vasodilatory, antioxidant, and anti-inflammatory properties (Kirkby et al. 2007; Mancuso and Barone 2009). Bilirubin-mediated reduction in oxidative stress and its cytoprotection effect have been demonstrated in the ischemia reperfusion injury model of the rat kidney (Adin et al. 2005). Mildly increased serum bilirubin level is related to a decreased risk for coronary artery disease and atherosclerosis in human (Mayer 2000) and is associated with decreased cancer mortality, resolution of asthmatic symptoms, and decreased incidence of retinopathy in prematurities (Heyman et al. 1989; Temme et al. 2001; Ohrui et al. 2003). Bilirubin is also known to have anti-inflammatory effects linked to its ability to scavenge free radicals (Hayashi et al. 1999; Stocker 2004; Mancuso et al. 2006a, 2006c, 2008).

Hyperglycemia and dyslipidemia increase oxidative stress in diabetes mellitus (Abraham and Kappas 2005). Oxidative injury to pancreatic \( \beta \)-cells causes insulin depletion and insulin resistance (Lenzen 2008). NADPH oxidase causes alterations in signal transduction, insulin secretion, insulin action, and cell proliferation in the diabetic condition (Newsholme et al. 2007), suggesting that it may be a target for intervention to improve insulin sensitivity in diabetes mellitus. The Gunn rat lacks the enzyme uridine-diphosphate glucuronosyltransferase that is responsible for conjugation and subsequent excretion of bilirubin into the bile, thus exhibiting elevation of plasma bilirubin (Pflueger et al. 2005). Considering the antioxidant and anti-inflammatory
properties of bilirubin, we hypothesized that hyperbilirubinemia may exert a protective effect on insulin resistance in diabetes mellitus by reducing oxidative stress. In this study, we employed homozygous Gunn rats to determine the effect of plasma unconjugated bilirubin on the initiation and progression of diabetes in streptozotocin (STZ)-injected animals.

**Materials and Methods**

**Animals**

Four-week-old male homozygous Gunn and control Wistar rats were obtained from Harlan (Indianapolis, IN, USA). All animals were fed standard rat chow and allowed free access to water. After a week of acclimatization, 12 Wistar and 10 Gunn rats are randomly divided into four groups (WC, $n = 7$; WS, $n = 5$; GC, $n = 5$; GS, $n = 5$). Following 6 h fasting, STZ (65 mg/kg body weight; Sigma, MO, USA) was injected to the rats (WS and GS groups) through intraperitoneal route. The rats in the WC and GC groups, as the control groups, were treated with the same volume of sodium citrate buffer. Approval for this study was obtained from the Seoul National University Budang Hospital Committee of animal experiment.

**Glucose measurement and HbA1c measurement**

Serum glucose level after 6-hour fasting was measured before and at 3, 7, 15, 21 and 28 days after STZ administration with a HemoCue B-glucose kit (HemoCue AB, Angelholm, Sweden) at the tail vein. Long-acting insulin (Lantus®, Aventis, Paris, France) had been given subcutaneously (2-4 IU/day) to the diabetic rats (5 rats in the WS group and 2 rats in the GS group) since 8 days after STZ administration, maintaining the non-fasting serum blood glucose level at around 400 mg/dL (Greg et al. 2007). The non-diabetic rats got normal saline instead of insulin. HbA1c levels were measured at 7 and 28 days after STZ injection by DCA 2000* HbA1c kit (Bayer, IN, USA).

**Oral glucose tolerance test**

Following overnight fasting, all rats were given 30% D-glucose solution (2 g/kg body weight) by gavage. Glucose levels were measured at 0, 30, 60, 90 and 120 minutes after gavage.

**Insulin ELISA assay**

The insulin levels were determined with a rat insulin enzyme-linked immunosorbent assay kit in pancreas lysate and culture media of insulinoma cells (Mercodia AB, Uppsala, Sweden).

**Plasma bilirubin measurement**

Total plasma bilirubin was measured colorimetrically using the Quantichrom bilirubin assay kit (DIBR-180, BioAssay Systems, Hayward, CA, USA).

**Measurement of reactive oxygen species (ROS) and nitric oxide (NO)**

The malondialdehyde concentration of plasma and urine was determined using the OxiSelect™ TBARS Assay Kit (Cell Biolabs, CA, USA). H$_2$O$_2$ production in the pancreas and in insulinoma cells was determined using DCFA-DA (2′,7′-dichlorofluorescin diacetate) (Sigma, MO, USA). Nitrate levels were measured using a Nitrate/Nitric Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA).

**Protein extraction and Western blotting**

The pancreas was homogenized and centrifuged, and proteins were extracted as previously described (Jadhav et al. 2008). The selected bands were scanned with an Imaging Densitometry instrument (GS-700, Bio-Rad, Hercules, CA, USA) and the density was determined with Molecular Analyst version 1.5 (Bio-Rad, Hercules, CA, USA).

**Severity of insulitis**

The pancreas sections were cut into 5-μm thick slices and stained with hematoxylin and eosin. Pancreatic islet histology was ranked according to an arbitrary scale, as described previously (Carlsson et al. 2000). The pancreatic sections were evaluated by a pathologist, unaware of the origin of the sections.

**Immunohistochemistry**

The expression levels of insulin and the NADPH oxidase components were evaluated. Tissue sections were deparaffinized and rehydrated through a graded series of alcohol to distilled water increments according to standard procedures. Endogenous peroxidase was blocked in a solution of 3% H$_2$O$_2$ in methanol for 10 min at room temperature. Non-specific staining was blocked with Dual Endogenous Enzyme Block (DakoCytomation, CA, USA) for 40 min at room temperature. Slides were incubated with anti-insulin (Santa Cruz Biotechnology, CA, USA), anti-NOX4, anti-p22phox, anti-p47phox, and anti-p67phox antibodies (all from Santa Cruz Biotechnology, CA, USA) antibodies overnight at 4°C, and detection was done using the DAB Detection Kit (DakoCytomation, CA, USA). For quantification, the numbers of stained cells and total cells per islet were counted (× 400) in 5 islets for each slide. The mean ratio of positive cells in the islets was calculated and the final results of each group were expressed as fold-changes compared to those of the WC group.

**Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining (TUNEL staining)**

TUNEL staining was performed on paraffin sections of the pancreas (5 μm) or the insulinoma cell using the in situ Cell Death Detection Kit (Fluorescein, Roche Diagnostics, Mannheim, Germany).

**Cell culture**

The insulinoma cell line (RIN-m5F) was purchased from ATCC and cultured using an ATCC-formulated RPMI-1640 Medium (catalog No. 30-2001, ATCC, USA) with fetal bovine serum to a final concentration of 10%. Cells (1 × 10$^5$ cells) were cultured in 6-well culture plate and incubated for 2 hours in the presence of STZ (2 mmol/L) with or without a 24-hr pre-treatment with bilirubin (Sigma, MO, USA) to a final concentration of 0.1 or 0.2 mg/dL dissolved in DMSO (Control, STZ-treated, and Pre-bil 0.1, Pre-bil 0.2 group). After that, the media was changed to the ATCC-formulated RPMI-1640 and the cell viability was determined by staining with trypan blue and insulin level in culture media was measured using ELISA kit (Mercodia AB, Uppsala, Sweden) at 2 or 4 hours after STZ treatment. H$_2$O$_2$ production was determined using DCFA-DA (Sigma, MO, USA). TUNEL staining (Fluorescein, Roche Diagnostics, Mannheim, Germany) to detect apoptotic cells was done at 2 hours after STZ treatment. We counted positive cells for TUNEL staining in 10 fields (× 400 magnification) in each culture well. All experiments were performed...
in triplicate.

Statistical analysis

The results are expressed as mean ± standard error. In the figures, the vertical bar indicates the confidence interval of mean. Statistical comparisons between animal groups were performed using SPSS (version 15.0; SPSS, IL, USA). Differences were analyzed by one-way ANOVA followed by Duncan’s post-hoc analysis or independent t-test depending on the number of groups. Statistical significance was set at a p value of < 0.05.

Results

The symptoms of STZ-induced diabetes mellitus were attenuated in Gunn rats

Before STZ injection, the characteristics of the control and STZ groups in each strain were not different but they were different between strains (Table 1). Gunn rats took less food and water and urinated less than did Wistar rats. The body weight and fasting glucose level were lower in Gunn rats than in Wistar rats. The serum total bilirubin was higher in Gunn rats (0.03 ± 0.01 vs. 4.78 ± 0.30 mg/dL, < 0.001). The grade of isletitis in the pancreas did not differ between the WC and WS groups or the GC and GS groups but is different between the WC and GC groups in baseline data, statistically.

All rats in the WS group (5/5 rats) developed diabetes, which was defined as fasting blood glucose 300 mg/dL or more, at 3 days, but only 2 of the 5 Gunn rats (GS group) became diabetic at 7 days after STZ injection. At 7 days after STZ administration without insulin injection, the fasting glucose level was higher in the WS group than in the GS group (WS : GS = 510.3 ± 50.3 : 236.4 ± 42.5 mg/dL, p = 0.001) and the HbA1c level was also higher in the WS group (WS : GS = 5.0 ± 0.1 : 3.9 ± 0.1, p = 0.001). The WS group showed typical diabetic features, loss of body weight, polyphagia, polydipsia, polyuria, and high level of serum cholesterol compared to the control group (WC group) (Table 1). The GS group had less severe diabetic symptoms than the WS group (Table 1). The 7 diabetic rats (5 and 2 from the WS and GS groups, respectively) had been injected with insulin to keep blood glucose around 400 mg/dL since 8 days after STZ injection, daily.

Gunn rats preserved insulin production in pancreatic islets regardless of STZ injection

Fasting blood glucose was raised in the WS group compared to the other groups (Fig. 1A). In the oral glucose tolerance test, the WS group showed the biggest area under the curve after glucose loading but the glucose intolerance was attenuated in the GS group (Fig. 1B). The hemoglobin A1c (HbA1c) was increased at 1 week and 4 weeks after STZ injection in the WS group compared to the WC group (at 4 weeks; 8.4 ± 0.4% vs. 3.6 ± 0.1%) (Fig. 1C). Although the HbA1c level of the GS group was increased compared to that of the GC group (at 4 weeks; 4.9 ± 0.3% vs. 3.4 ± 0.1%), it was lower than that of the WS group (p < 0.001). Plasma insulin level was the lowest in the WS group at 4 weeks (WC : WS : GC : GS = 87.1 ± 15.3 : 35.7 ± 2.5 : 80.2 ± 9.6 : 62.2 ± 6.3 pM/L, p = 0.041) (Fig. 1D).

The grade of isletitis in the pancreas did not differ between the WS and GS groups (data not shown) but the STZ-induced decrease in insulin production was the greatest in WS group (Fig. 2). STZ induced apoptosis in the islet cells of the Wistar rats (the percentage of apoptotic cells to total cells in islets; 1.7 ± 0.9% and 82.7 ± 2.0% in the WS and WS groups, respectively, p < 0.001). However, in Gunn rats, the proportion of apoptosis was not different between the GC and GS groups (40.9 ± 3.0% and 44.6 ± 1.8% in the GC and GS groups, respectively, p = 0.309) (Fig. 3).

Table 1. The characteristics before and after STZ treatment of the animals.

<table>
<thead>
<tr>
<th>Baseline data</th>
<th>WC (n = 7)</th>
<th>WS (n = 5)</th>
<th>GC (n = 5)</th>
<th>GS (n = 5)</th>
<th>p value</th>
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<tr>
<td>Body weight (g)</td>
<td>144.9 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>143.3 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105.4 ± 4.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>103.6 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
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<td>Water intake (ml/day)</td>
<td>29.4 ± 4.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.4 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.9 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.6 ± 4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
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<td>Food intake (ml/day)</td>
<td>11.9 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.4 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.1 ± 2.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.5 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.140</td>
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<tr>
<td>Urine volume (ml/day)</td>
<td>18.6 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.3 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.5 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.6 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
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<td>FBS (mg/dL)</td>
<td>148 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>159 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>135 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>139 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
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<th>At 4 weeks after STZ</th>
<th>WC (n = 7)</th>
<th>WS (n = 5)</th>
<th>GC (n = 5)</th>
<th>GS (n = 5)</th>
<th>p value</th>
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<tr>
<td>Body weight (g)</td>
<td>317 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>234 ± 12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>182 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
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<td>Water intake (ml/day)</td>
<td>21.1 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>132.1 ± 12.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.3 ± 7.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Food intake (ml/day)</td>
<td>12.0 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.2 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.1 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.1 ± 2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
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<tr>
<td>Urine volume (ml/day)</td>
<td>13.1 ± 2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>114.3 ± 7.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.7 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.0 ± 5.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
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<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.02 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.72 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.83 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
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<td>Serum cholesterol (mg/dL)</td>
<td>64 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91 ± 9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Weight of pancreas (g)</td>
<td>0.24 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.29 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.36 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
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WC, Wistar rat injected with vehicle; WS, Wistar rat injected with streptozotocin (65 mg/kg/day, ip, once); GC, Gunn rat injected with vehicle; GS, Gunn rat injected with streptozotocin (65 mg/kg/day, ip, once). Data expressed as mean ± standard error, Groups marked with the same letter are not different by post-hoc analysis with Duncan test. For example, the body weight is not different between the WC and WS groups or the GC and GS groups but is different between the WC and GC groups in baseline data, statistically.
Fig. 1. The resistance of Gunn rats to damage by STZ injection. STZ injection elevated fasting glucose and HbA1c levels and impaired glucose tolerance in Wistar rats but, in hyperbilirubinemic Gunn rats, those changes were decreased. A. Fasting blood glucose level after injection of STZ. B. Oral glucose tolerance test at 4 weeks after injection of STZ. C. HbA1c levels at 1 week and 4 weeks after injection of STZ. D. Plasma insulin level at 4 weeks after injection of STZ. The vertical bar indicates the 95% confidence interval of the mean in each group. Note that groups marked with the same letter are not significantly different. *p < 0.05, difference from WC group, #p < 0.05 difference between the WS and GS groups, WC: Wistar control (n = 7), WS: Wistar treated with STZ (n = 5), GC: Gunn control (n = 5), and GS: Gunn treated with STZ (n = 5).

Fig. 2. Insulin production in the pancreas. The insulin production in the islets stained with anti-insulin antibody in WS group was greatly decreased by STZ but, in the GS group, was more preserved compared to the WS group (× 400). A. Wistar control, B. Wistar treated with STZ, C. Gunn control, and D. Gunn treated with STZ. E. The percent of insulin producing cells in islets was calculated in 5 islets per each rat and was minimized in the WS group. The vertical bar indicates the 95% confidence interval of the mean in each group. WC: Wistar control (n = 7), WS: Wistar treated with STZ (n = 5), GC: Gunn control (n = 5), and GS: Gunn treated with STZ (n = 5). Groups marked with the same letter are not significantly different.
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The increase of NO production in tissues by STZ was attenuated in Gunn rats

In Gunn rats, amount of pancreas nitrate (reflective of NO production) was not changed by STZ injection but was significantly increased in Wistar rats (WC : WS : GC : GS = 5.94 ± 1.44 : 11.97 ± 1.18 : 5.48 ± 0.77 : 6.62 ± 1.67 μM/500 μg protein, p = 0.015).

STZ increased the expressions of NADPH oxidase and oxidative stress in the Wistar rats but not in Gunn rats

The expression of GLUT2 protein in the pancreas, through which the STZ moves into the islet cell, was not different between Wistar and Gunn rats (Fig. 4). In the pancreas islets of the WS group, the expression levels of NOX4, p22phox, and p67phox were elevated by STZ (Fig. 5). The expressions of NOX4, p22phox, and p67phox in the pancreas tissue of the WS group were increased by 2.23 ± 0.26, 2.52 ± 0.19, and 2.18 ± 0.15 folds, compared to the WC group, respectively.

At 4 weeks after STZ injection, the H2O2 production in the pancreas was increased the most in the WS group (WC : WS : GC : GS = 24.2 ± 0.9 : 30.2 ± 2.5 : 22.6 ± 0.8 : 22.8 ± 0.9 arbitrary unit with DFCA-DA, expressed as unit/mg protein, p = 0.006).

Bilirubin pre-treatment decreased the STZ-mediated death, apoptosis, and H2O2 production in insulinoma cells

In insulinoma cell culture, bilirubin decreased cell death by STZ (Fig. 6A). With 0.1 mg/dL bilirubin, cells were well preserved until 4 hours after STZ treatment, but with 0.2 mg/dL bilirubin, the effect was evident only during 2 hours. The insulin level in the culture media was the highest in the STZ-treated cells (Fig. 6B). Among cells harvested 2 hours after STZ treatment, the number of TUNEL positive cells (Control : STZ-treated : Pre-bil 0.1 : Pre-bil 0.2 = 6.5 ± 2.3 : 10.1 ± 2.0 : 7.2 ± 2.2 : 7.4 ± 2.4,
cell numbers/field (× 400), \( p = 0.005 \) and the level of \( \text{H}_2\text{O}_2 \) production (Control : STZ-treated : Pre-bil, 0.1 : Pre-bil, 0.2 = 9.7 ± 1.6 : 13.2 ± 0.9 : 6.2 ± 1.2 : 8.7 ± 2.3, units/10^5 cells, \( p < 0.001 \)) were the highest in the STZ-treated cells.

**Discussion**

Our findings demonstrated the resistance of the hyperbilirubinemic Gunn rats to STZ-induced pancreatic damage with the decrease of the activation of NADPH oxidase, generation of oxidative stress, and cellular apoptosis in the pancreas. STZ, taken up via GLUT2, induced cell death mainly through alkylation of DNA in target tissues (Bennett and Pegg 1981), which is related to NO generation during the STZ metabolic process (Pflueger et al. 2005). The STZ-induced pancreatic damage could be reduced with NO scavengers (Kröncke et al. 1995). ROS also contributed to DNA fragmentation and STZ-induced cell changes, which was generated from mitochondria and xanthine oxidase (Nakatsuka et al. 1990). Compared to Wistar rats, STZ injection failed to induce diabetes and pancreatic damage in Gunn rats, although there was no difference of GLUT2 expression in the pancreas between the two strains. One of the possible mechanisms is the role of bilirubin as an endogenous NO scavenger (Wang et al. 2004). The STZ-induced NO production in the pancreas was decreased in Gunn rats compared to Wistar rats. In previous reports, NO donors, such as S-nitroscysteine and S-nitrosothioglutathione, reduced the half-life of bilirubin through direct interaction between bilirubin and NO (Mancuso et al. 2003, 2006b). Bilirubin scavenged a secondary oxidant of peroxynitrite through a hydrogen donation mechanism (Minetti et al. 1998; Barone et al. 2009), which is an extremely potent and stable oxidant formed by the interaction of NO and superoxide anions (Kaur et al. 2003), and bilirubin also inhibited NO production in animals treated with lipopolysaccharide (Wang et al. 2004; Mancuso et al. 2006c, 2008).

Bilirubin’s role as an antioxidant depended on the inhibition of the membrane-bound NADPH oxidase (Matsumoto et al. 2006). Bilirubin inhibited the assembly of NADPH oxidase subunits, such as membrane translocation of p47phox (Matsumoto et al. 2006). The pancreas islets expressed the NADPH oxidase components, NOX1, NOX2, NOX4, and p22phox, as membrane-associated components, and p47phox, NOX01, p40phox, and p67phox as cytosolic components (Nakayama et al. 2005; Uchizono et al. 2006). Hyperglycemia increased superoxide production and the expression of p47phox and p22phox in a rat, insulin-producing cell lines, and isolated human pancreatic islets (Calabrese et al. 2007). In type 2 diabetic animals, NOX2 and p22phox were significantly increased in the pancreas islets and angiotensin II type 1 receptor agonist attenuated the increased expression and partially restored the decreased insulin contents in islets (Nakayama et al. 2005). In the early phase of diabetes in NOD mice, the upregulation of heme oxygenase-1, a key enzyme in bilirubin production, delayed the development of diabetes by decreasing p47phox and superoxide generation and increasing antiapoptotic signaling protein (Li et al. 2007). We confirmed that the STZ-induced pancreas destruction in the Wistar rats was associated with increased expressions of NOX4, p22phox, and p67phox, and an increased level of \( \text{H}_2\text{O}_2 \), which were attenuated in the Gunn rats. This phenomenon was also evident when we compared the expressions of NADPH oxidase components between the 5 diabetic Wistar rats and 2 diabetic Gunn rats. Considering bilirubin’s role as an NO scavenger and inhibitor of NADPH oxidase to decrease ROS production, the hyperbilirubinemic Gunn rat could be resistant to the STZ-induced pancreas islets destruction.

Bilirubin also decreased cell death, apoptosis, and \( \text{H}_2\text{O}_2 \) production in insulinoma cell culture. Bilirubin is both cytoprotective and cytotoxic. Bilirubin quenches ROS and inhibits inflammatory and mitogen-induced ROS-mediated responses, but its’ elevated level in the newborn adversely affects neuronal cells (Wang et al. 2004). We tried variable concentrations of bilirubin in cell culture experiments (0.1, 0.2, 0.3, 0.5, 1, and 3 mg/dL of bilirubin) and found that 0.1-0.2 mg/dL of unconjugated free bilirubin could be protective for the STZ-induced insulinoma cell.
Fig. 5. Expression of NADPH oxidase subunits in the pancreas. Shown are NOX4 (A), p22phox (B), p47phox (C) and p67phox (D) in islets. The increased expression of NOX4, p22phox, and p67phox by STZ was suppressed by hyperbilirubinemia, as evaluated by immunohistochemistry staining (× 400). Immunohistochemistry staining quantification is expressed as fold-changes compared to the WC group for the proportion of cells stained with antibodies to NOX4 (E), p22phox (F), p47phox (G) and p67phox (H). The proportion of cells stained with antibodies to total cells in an islet was counted in 5 islets of each rat. The vertical bar indicates the 95% confidence interval of the mean in each group. Groups marked with the same letter are not different by post-hoc analysis with Duncan test. WC: Wistar control (n = 7), WS: Wistar treated with STZ (n = 5), GC: Gunn control (n = 5), and GS: Gunn treated with STZ (n = 5).
damage. The cell viability measured by tryptophan blue staining and the apoptosis judged by TUNEL staining were preserved in cells pre-treated with bilirubin. The insulin concentration in the culture media, which was secreted or released from cell lysis, was the lowest in cells treated with bilirubin before STZ.

In conclusion, the present study demonstrated the effects of hyperbilirubinemia in Gunn rats against STZ-induced pancreatic damage. We postulate that inhibition of NADPH oxidase subunits, resulting in decreased ROS and NO production in the pancreas, may be involved in the mechanism by which bilirubin exerts its beneficial role on the pancreas in the Gunn rat. These results support the potential of bilirubin as a therapeutic modality for diabetes. Further studies will be necessary to confirm this potential.

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Statement of competing financial interests
There is no conflict of interest.

References

Fig. 6. Bilirubin attenuated insulinoma cell death by STZ.
The mean proportion of live insulinoma cells in each group compared to control group was the lowest in the streptozotocin (STZ)-treated group. The cell viability was measured by trypan blue method. The insulin level in the culture media of 1 × 10^6 cells was the highest in the STZ-treated group. A: Ratio of viable cells. B: Concentration of insulin in culture media. *difference between each group and STZ-treated group, p < 0.05. C: control group, S: STZ-treated group, B1: cells pretreated with 0.1 mg/dL bilirubin 24 hours before STZ treatment, B2: cells pretreated with 0.2 mg/dL bilirubin 24 hours before STZ treatment. At 2 hours: cells harvested 2 hours after STZ treatment. At 4 hours: cells harvested 4 hours after STZ treatment.