Hyperbilirubinemia, Augmentation of Endothelial Function and Decrease in Oxidative Stress in Gilbert Syndrome

Running title: Maruhashi et al.; Endothelial function in Gilbert syndrome

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Abstract:

**Background** - Patients with Gilbert syndrome have mild unconjugated hyperbilirubinemia. It has been shown that bilirubin is an endogenous antioxidant. We evaluated the role of oxidative stress in endothelial function in patients with Gilbert syndrome under normal conditions without cardiovascular risk factors.

**Methods and Results** - A total of 108 young men with Gilbert syndrome without cardiovascular risk factors and 108 age-matched healthy men (normal controls) were enrolled in this study. Serum concentrations of bilirubin were higher in patients with Gilbert syndrome than in control subjects (29.2±11.6 vs. 9.4±2.7 µmol/L, P<0.001). Serum concentrations of malondialdehyde-modified low-density lipoprotein (MDA-LDL) and urinary excretion of 8-hydroxy-2’-deoxyguanosine (8-OHdG), as indices of oxidative stress, were lower in patients with Gilbert syndrome than in control subjects (61.8±24.5 vs. 72.5±21.8 U/L, P=0.034, 7.8±2.4 vs. 10.4±3.2 ng/mg creatinine, P=0.001, respectively). Flow-mediated vasodilation (FMD) was greater in patients with Gilbert syndrome than in normal control subjects (7.2±2.2% vs. 5.9±1.7%, P<0.001). Vascular responses to nitroglycerine were not significantly different between the two groups. FMD correlated with serum concentration of bilirubin (r=0.44, P<0.001), MDA-LDL (r=−0.25, P=0.01), and urinary excretion of 8-OHdG (r=−0.27, P=0.004) in patients with Gilbert syndrome but not in control subjects. In addition, serum concentration of bilirubin correlated with MDA-LDL (r=−0.20, P=0.04) and 8-OHdG (r=−0.21, P=0.02) in patients with Gilbert syndrome but not in control subjects.

**Conclusions** - Patients with Gilbert syndrome had low levels of oxidative stress associated with hyperbilirubinemia and enhancement of endothelium-dependent vasodilation.

**Clinical Trial Registration Information** - [http://www.umin.ac.jp](http://www.umin.ac.jp); Identifier: UMIN000003409.

**Key words:** bilirubin; endothelial function; oxidative stress; Gilbert syndrome
Introduction

Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, leading to cardiovascular complications.\(^1\) Oxidative stress also plays an important role in the pathogenesis and development of cardiovascular diseases.\(^2,3\) Normal endothelial function is maintained by a balance of oxidative stress and nitric oxide (NO).\(^4,5\) One mechanism of endothelial dysfunction is an increase in oxidative stress, which inactivates NO. Indeed, we have shown that inactivation of the renin-angiotensin system, particularly angiotensin II, by successful renal angioplasty decreases oxidative stress, resulting in improvement of endothelium-dependent vasodilation in patients with renovascular hypertension, who are ideal subjects for determining how endothelial function is affected by excess angiotensin II and angiotensin II-related increase in oxidative stress through the activation of NADPH oxidase.\(^6\)

Patients with Gilbert syndrome have mild unconjugated nonhemolytic hyperbilirubinemia and the incidence of Gilbert syndrome in the general population is 5% to 10%.\(^7-9\) Although bilirubin at a high concentration acts as a cytotoxic metabolite, bilirubin at a low concentration is a potent endogenous antioxidant.\(^10-14\) Indeed, Inoguchi et al.\(^15\) showed that oxidative stress markers were decreased in patients with diabetes who had Gilbert syndrome compared with those in patients with diabetes who did not have Gilbert syndrome. Therefore, patients with Gilbert syndrome are ideal models for determining how endothelium-dependent vasodilation is affected by bilirubin-induced decrease in oxidative stress. Evaluation of endothelial function in patients with Gilbert syndrome would enable more specific conclusions to be drawn regarding the role of oxidative stress in endothelial function.

To determine the role of the decrease in oxidative stress in endothelial function, we evaluated flow-mediated vasodilation (FMD) and oxidative stress markers in young patients with
Gilbert syndrome under normal conditions without cardiovascular risk factors.

Methods

Subjects

A total of 108 young men with Gilbert syndrome without cardiovascular risk factors and 108 age-matched healthy men (normal controls) were enrolled from the 11922 subjects of the databases of Rho-associated Kinase Study Registry and Flow-mediated Dilation Japan Registry. Gilbert syndrome was diagnosed by the following criteria: mild unconjugated hyperbilirubinemia (1.2 to 6.0 mg/dL [20 to 103 μmol/L]), normal values of hepatic biochemical tests, absence of liver disease or overt hemolysis, UDP-glucuronosyltransferase 1 activity of less than 35% of normal, and results of real-time PCR detection of UDP-glucuronosyltransferase 1*28 mutation.16,17 Crigler-Najjar, Dubin-Johnson, and Rotor syndromes were also excluded. In normal control subjects, the upper limit of the normal range for serum concentrations of bilirubin was 1.0 mg/dL (17.1 μmol/L). None of the subjects had a history of cardiovascular or cerebrovascular disease or of renal disease. The subjects took no medication for at least 12 weeks before the study. Women were excluded from this study because of potential confounding factors for assessing vascular function, including menstrual cycle. The ethical committees of our institutes approved the study protocol. Written informed consent for participation in the study was obtained from all of the subjects.

Measurement of FMD

All studies were performed in the morning, after overnight fasting, in a quiet, dark, air-conditioned room (constant temperature of 22-25°C). The subjects remained supine throughout the study. The vascular response to reactive hyperemia in the brachial artery was assessed for ultrasound assessment of endothelium-dependent FMD. A high-resolution linear artery
transducer was coupled to computer-assisted analysis software (UNEXEF18G, UNEX Co., Nagoya, Japan) that used an automated edge detection system for measurement of brachial artery diameter. A blood pressure cuff was placed around the forearm. The brachial artery was scanned longitudinally 5-10 cm above the elbow. When the clearest B-mode image of the anterior and posterior intimal interfaces between the lumen and vessel wall was obtained, the transducer was held at the same point throughout the scan by a special probe holder (UNEX Co.) to ensure consistency of the image. Depth and gain setting were set to optimize the images of the arterial lumen wall interface. When the tracking gate was placed on the intima, the artery diameter was automatically tracked and the waveform of diameter changes over the cardiac cycle was displayed in real time using the FMD mode of the tracking system. This allowed the ultrasound images to be optimized at the start of the scan and the transducer position to be adjusted immediately for optimal tracking performance throughout the scan. Pulsed Doppler flow was assessed at baseline and during peak hyperemic flow, which was confirmed to occur within 15 seconds after cuff deflation. Blood flow velocity was calculated from the color Doppler data and was displayed as a waveform in real time. The baseline longitudinal image of the artery was acquired for 30 seconds and then the blood pressure cuff was inflated to 50 mm Hg above systolic pressure for 5 minutes. The longitudinal image of the artery was recorded continuously until 5 minutes after cuff deflation. Pulsed Doppler velocity signals were obtained for 20 seconds at baseline and for 10 seconds immediately after cuff deflation. Changes in brachial artery diameter were immediately expressed as percent change relative to the vessel diameter before cuff inflation. FMD was automatically calculated as the percent change in peak vessel diameter from the baseline value. \%FMD (peak diameter - baseline diameter/baseline diameter) was used for analysis. Blood flow volume was calculated by multiplying the Doppler flow velocity
(corrected for the angle) by heart rate and vessel cross-sectional area (-r2). Reactive hyperemia was calculated as the maximum percentage increase in flow after cuff deflation compared with baseline flow.

The response to nitroglycerine was used for assessment of endothelium-independent vasodilation. After acquiring baseline rest image for 30 seconds, a sublingual tablet (nitroglycerine 75 µg) was given, and image of the artery was recorded continuously for 5 minutes. Nitroglycerine-induced vasodilation was automatically calculated as a percent change in peak vessel diameter from the baseline value. %nitroglycerine (peak diameter - baseline diameter/baseline diameter) was used for analysis.

The observers were blind to the form of examination.

**Analytical Methods**

Routine chemical methods were used to determine serum concentrations of bilirubin, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, creatinine, glucose, and electrolytes. The serum concentrations of malondialdehyde-modified low-density lipoprotein (MDA-LDL) were assayed by ELISA (anti-MDA-LDL antibody, SRL Co., Atsugi, Japan). Serum concentrations of high-sensitivity C-reactive protein (hs-CRP) were measured by a high sensitive nephelometry assay using a CRP kit (Dade Behring, Deerfield, IL). Serum concentrations of interleukin-6 were measured by a high sensitivity enzyme-linked immunosorbent assay (ELISA) (R&D System, Minneapolis, MN). The urinary concentrations of 8-hydroxy-2’-deoxyguanosine (8-OHdG) were assayed by ELISA using 8-OHdG kits (Nihon Yushi Co., Tokyo, Japan).

**Statistical Analysis**

Results are presented as mean ± SD. All reported p values were 2-sided, and a p value of <0.05
was considered statistically significant. We created matched pairs (1 Gilbert syndrome patient: 1 control). Paired $t$ test was used for comparison of mean values of continuous variables between the two groups. Correlations between continuous variables were estimated using Spearman correlation coefficients. The data were processed using the software package Stata version 9 (Stata Co., College Station, TX).

**Results**

**Clinical characteristics**

Clinical characteristics of patients with Gilbert syndrome and control subjects are summarized in Table 1. Serum concentrations of bilirubin were significantly higher in patients with Gilbert syndrome than in control subjects. Serum concentrations of MDA-LDL and urinary excretion of 8-OHdG, as indices of oxidative stress, were significantly lower in patients with Gilbert syndrome than in control subjects. There was no significant difference in other parameters between the two groups.

**Vascular function in Gilbert syndrome**

FMD was significantly greater in patients with Gilbert syndrome than in normal control subjects (7.2±2.2% vs. 5.9±1.7%, P<0.001) (Figure 1). Brachial artery diameter at baseline and increase in hyperemic blood flow were similar in the two groups (Table 2). Vascular responses to nitroglycerine were not significantly different between the two groups (Table 2).

**Relationships between bilirubin, oxidative stress markers and FMD**

FMD significantly correlated with serum concentration of bilirubin ($r=0.44$, $P<0.001$) (Figure 2, top), serum concentration of MDA-LDL ($r=-0.25$, $P=0.01$), and urinary excretion of 8-OHdG ($r=-0.27$, $P=0.004$) in patients with Gilbert syndrome but not in control subjects. In addition,
serum concentration of bilirubin significantly correlated with serum concentration of MDA-LDL (r=−0.20, P=0.04) (Figure 2, middle) and urinary excretion of 8-OHdG (r=−0.21, P=0.02) in patients with Gilbert syndrome (Figure 2, bottom) but not in control subjects. There were no significant relationships between vascular responses to nitroglycerine and bilirubin, MDA-LDL and 8-OHdG in the two groups.

Discussion

Our findings demonstrate that patients with Gilbert syndrome have low levels of oxidative stress associated with hyperbilirubinemia and enhancement of endothelium-dependent vasodilation. These beneficial effects on the vasculature may contribute to reduced prevalence of vascular complications in atherosclerotic patients with Gilbert syndrome compared with that in atherosclerotic patients without Gilbert syndrome.15,18

The purpose of this study was to determine the role of decrease in oxidative stress per se in endothelial function under a normal condition without cardiovascular risk factors in humans. Therefore, we selected healthy young men to avoid the possibility of alteration in endothelial function caused by confounding factors, including hypertension, heart failure, atherosclerosis, dyslipidemia, diabetes mellitus, smoking, aging, and menstrual cycle. Bilirubin-induced decrease in oxidative stress augmented endothelium-dependent vasodilation in healthy young male patients with Gilbert syndrome, suggesting that reactive oxygen species (ROS) even under a normal condition are a predictor of endothelial function.

Several studies using atherosclerotic animal models and patients with atherosclerosis have shown that endothelial dysfunction is associated with an increase in ROS.19-21 A balance between ambient levels of superoxide and NO release plays a critical role in the maintenance of
normal endothelial function. Both 8-OHdG and MDA-LDL have been used as indices of oxidative stress. 8-OHdG is one of the most common markers for evaluating oxidative DNA damage and is a product formed by specific attack of a hydroxy radical on DNA. Measurement of MDA-LDL has been proposed as the biologic signature of clinical in vivo LDL oxidation. In the present study, urinary 8-OHdG excretion and serum MDA-LDL concentration were significantly lower in patients with Gilbert syndrome than in controls. In addition, there were significant relationships of FMD with urinary 8-OHdG excretion and serum MDA-LDL concentration. These findings suggest that inhibition of NO inactivation by decrease in production of ROS may contribute to augmentation of endothelial function in patients with Gilbert syndrome.

In the present study, oxidative stress markers decreased in relation to increase in serum concentration of bilirubin in patients with Gilbert syndrome. There was a significant relationship between FMD and serum concentration of bilirubin. The mechanism by which endothelial function was augmented in patients with Gilbert syndrome may be due to the bilirubin-induced decrease in oxidative stress. Serum bilirubin should be one of the key mediators of the antioxidant system in human.

Bilirubin is a metabolic end product of heme degradation by heme oxygenases, especially heme oxygenase-1, which converts heme to biliverdin, and then biliverdin is reduced to bilirubin by biliverdin reductase. In 1987, Stocker et al. for the first time demonstrated that bilirubin scavenged peroxyl radicals more effectively than did the powerful antioxidant α-tocopherol in vitro. Since then, several investigators have shown that bilirubin has antioxidant effects, including inhibition of membrane lipid peroxidation and scavenging of ROS. It is well known that the major source of ROS in the vasculature is an NADPH oxidase activation that
is induced by various stimuli.\textsuperscript{27,28} We have reported that endothelial function is restored by inhibition of angiotensin II-induced NADPH oxidase activity in a clinical setting.\textsuperscript{6} Interestingly, bilirubin inhibits NADPD oxidase activity \textit{in vitro} and \textit{in vivo},\textsuperscript{29,30} suggesting that production of ROS is decreased by bilirubin. Oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases.\textsuperscript{2,3} Clinical studies have shown that serum concentrations of bilirubin inversely correlate with risk of cardiovascular diseases and peripheral arterial disease.\textsuperscript{31-33} In addition, the prevalence of ischemic heart disease in patients with Gilbert syndrome was only 2\%, much lower than the prevalence of 12\% in the general population.\textsuperscript{18} These findings suggest that bilirubin is a potent endogenous antioxidant and has beneficial effects on inhibition of the development of atherosclerosis, probably due to its ability of antioxidation.

Chronic inflammation may also contribute to endothelial dysfunction through a decrease in NO bioavailability, a decrease in NO production and/or an increase in NO inactivation. It is known that bilirubin exerts anti-inflammatory effects on the vasculature.\textsuperscript{34} However, in the present study, there were no significant differences in serum levels of interleukin-6 and hs-CRP between patients with Gilbert syndrome and controls, suggesting that inflammation is not decreased in patients with Gilbert syndrome. It is unlikely that augmentation of endothelial function is due to decrease in inflammation in patients with Gilbert syndrome who do not have cardiovascular risk factors.

Values of FMD and nitroglycerine-induced vasodilation in healthy young adults in the present study may be lower than those reported by other groups, for healthy young adults. Although we do not know the precise reasons for the variability of values of FMD and nitroglycerine-induced vasodilation, existence of variability in the values of FMD and nitroglycerine-induced vasodilation may be due to the differences in methods for measurement.
of FMD and nitroglycerine-induced vasodilation (e.g., differences in testing modality, position of
cuff placement, timing of measurement of vasodilation, and skill of the observer).

It is known that human immunodeficiency virus protease inhibitor atazanavir increases
bilirubin levels through an inhibition of UDP-glucuronosyltransferase 1 activity.\textsuperscript{35} Dekker et al.\textsuperscript{36} have shown that an increased level of bilirubin induced by atazanavir improves endothelium-
dependent vasodilation in patients with type 2 diabetes mellitus, supporting our findings.
However, in contrast, Dube et al.\textsuperscript{37} found no change in endothelial function in healthy subjects
treated with atazanavir despite an increase in bilirubin level. Although we do not know the
precise reasons for the discrepancy of these results, some explanations are postulated: difference
in subject selection (diabetes mellitus vs. healthy subjects), difference in increase in average
bilirubin levels induced by atazanavir (3.8 mg/dL vs. 1.2 mg/dL), difference in dose of atazanavir
used (600 mg/d vs. 400 mg/d), difference in treatment period (3 days vs. 4 weeks), and a
relatively small number of subjects. In addition, there is a clear difference between our study and
previous studies: subjects in our study were patients with Gilbert syndrome, whereas subjects in
previous studies were models of hyperbilirubinemia induced by atazanavir.

In conclusion, a balance between ambient levels of superoxide and NO release plays a
critical role in the maintenance of endothelial function. Bilirubin is a potent antioxidant and a
mediator of endothelial function through inhibition of NO inactivity by its antioxidative effect.
When measuring endothelial function, we should take care about the existence of Gilbert
syndrome, since Gilbert syndrome is relatively common in the general population. Endothelial
function may be overestimated in healthy subjects with Gilbert syndrome or hyperbilirubinemia.
It is likely that superoxide plays an important role in vascular function even under a normal
condition without cardiovascular risk factors.
Acknowledgments: We thank Megumi Wakisaka, Kiichiro Kawano and Satoko Michiyama for their excellent secretarial assistance.

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Conflict of Interest Disclosures: None.

References:


Table 1. Clinical Characteristics of the Control Subjects and the Patients with Gilbert Syndrome

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control subjects (n=108)</th>
<th>Gilbert syndrome (n=108)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>23±5</td>
<td>23±5</td>
<td>0.98</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.5±4.3</td>
<td>22.9±4.3</td>
<td>0.85</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>112.2±9.4</td>
<td>111.6±8.8</td>
<td>0.72</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>72.1±8.2</td>
<td>71.4±7.9</td>
<td>0.69</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>66.4±9.6</td>
<td>68.4±8.7</td>
<td>0.61</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.51±0.98</td>
<td>4.34±1.12</td>
<td>0.23</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.33±0.69</td>
<td>1.29±0.71</td>
<td>0.21</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.38±0.48</td>
<td>1.46±0.43</td>
<td>0.26</td>
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<td>LDL cholesterol, mmol/L</td>
<td>2.26±0.77</td>
<td>2.41±0.58</td>
<td>0.73</td>
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<tr>
<td>Glucose, mmol/L</td>
<td>4.8±0.5</td>
<td>4.8±0.6</td>
<td>0.92</td>
</tr>
<tr>
<td>Bilirubin, μmol/L</td>
<td>9.4±2.7</td>
<td>29.2±11.6</td>
<td>&lt;0.001</td>
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<tr>
<td>Interleukin-6, ng/L</td>
<td>1.1±2.1</td>
<td>1.0±1.9</td>
<td>0.22</td>
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<td>High-sensitivity CRP, mg/L</td>
<td>0.58±0.31</td>
<td>0.53±0.29</td>
<td>0.18</td>
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<tr>
<td>MDA-LDL, U/L</td>
<td>2.5±21.8</td>
<td>61.8±24.5</td>
<td>0.032</td>
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<tr>
<td>Urinary excretion of 8-OHdG, ng/mg creatinine</td>
<td>10.4±3.2</td>
<td>7.8±2.4</td>
<td>0.001</td>
</tr>
</tbody>
</table>

HDL-C indicates high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; MDA-LDL, malondialdehyde-modified low-density lipoprotein; 8-OHdG, 8-hydroxy-2’-deoxyguanosine.

All results are presented as mean±SD.

Table 2. Vascular function of the Patients with Gilbert Syndrome and the Control Subjects

<table>
<thead>
<tr>
<th>Vascular function</th>
<th>Control subjects (n=108)</th>
<th>Gilbert syndrome (n=108)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial artery diameter, mm</td>
<td>3.78±0.42</td>
<td>3.82±0.45</td>
<td>0.78</td>
</tr>
<tr>
<td>Increased hyperemic blood flow, %</td>
<td>378±214</td>
<td>345±245</td>
<td>0.63</td>
</tr>
<tr>
<td>Nitroglycerine-induced vasodilation, %</td>
<td>12.0±2.3</td>
<td>11.8±3.2</td>
<td>0.45</td>
</tr>
</tbody>
</table>

All results are presented as mean±SD.
Figure Legends:

**Figure 1.** Bar graphs show flow-mediated vasodilation (FMD) to hyperemic flow in control subjects and patients with Gilbert syndrome.

**Figure 2.** Scatter plots show significant correlations between serum concentration of bilirubin and flow-mediated vasodilation (FMD) ($r=0.44$, $P<0.001$, top), serum concentration of malondialdehyde-modified low-density lipoprotein (MDA-LDL) ($r=-0.20$, $P=0.04$, middle) and urinary excretion of 8-hydroxy-2’-deoxyguanosine (8-OHdG) ($r=-0.21$, $P=0.02$, bottom) in patients with Gilbert syndrome (closed circle) but not in control subjects (open circle).
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