High Serum Bilirubin Concentrations Preserve Coronary Flow Reserve and Coronary Microvascular Functions

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Background—Elevated serum bilirubin concentrations protect against atherosclerotic diseases; however, it is not clear whether higher serum bilirubin concentrations in physiological ranges work in favor of the cardiovascular system in younger persons with no cardiovascular risk factors. Accordingly, we investigated the effects of high, intermediate, and low serum bilirubin concentrations on coronary flow reserve (CFR).

Methods and Results—Fifty-two healthy subjects with hyperbilirubinemia (total bilirubin 1.43±0.33 mg/dL; mean age 35.9±7.3), 55 subjects with intermediate bilirubin level (total bilirubin: 0.69±0.11 mg/dL; mean age: 36.4±6.8), and 53 healthy subjects with hypobilirubinemia (total bilirubin 0.37±0.08 mg/dL; mean age, 37.6±6.6) were studied. Transthoracic second harmonic Doppler echocardiography examination was performed using an Acuson Sequoia C256 Echocardiography System. Coronary diastolic peak flow velocities were measured at baseline and after dipyridamole infusion (0.84 mg/kg over 6 minutes). CFR was calculated as the ratio of hyperemic to baseline diastolic peak velocities. Demographic features, coronary risk factors, echocardiographic measurements, and biochemical measurements were similar among the 3 groups, except high-sensitivity C-reactive protein (hsCRP). CFR values were significantly higher in subjects with high bilirubin concentrations than those in the intermediate and the low bilirubin groups (3.19±0.73; 2.75±0.42; 2.56±0.52, respectively; P<0.0001), and hsCRP levels were significantly lower in subjects with high bilirubin concentrations than those in both intermediate and low bilirubin groups (1.4±1.0, 2.0±1.7, 3.0±1.9 mg/L, respectively; P<0.001). hsCRP levels correlated with total bilirubin concentration and with CFR.


Key Words: coronary flow reserve ■ bilirubin ■ echocardiography

It is generally accepted that development of atherosclerosis involves several oxidative reactions such as formation of oxygen and peroxyl radicals, and lipid oxidation, especially oxidation of low-density lipoprotein (LDL) cholesterol.1,2 This understanding of the pathogenesis of atherosclerosis has rendered the antioxidants important as means of preventing atherosclerosis and related diseases. Because oxidative modification of LDL results from lipid peroxidation, water and lipid-soluble antioxidants should have a prominent effect in preventing this modification.3 Accordingly, several antioxidants such as probucol, phenothiazines, calcium antagonists, agents complexing copper and iron ions, vitamins A, C, and E,4,5 and bilirubin6–11 have been demonstrated as having a role in preventing oxidative modification. Bilirubin is a major product of hem catabolism. Previously published several studies have demonstrated the relationship between serum bilirubin levels and oxidative stress-mediated diseases, especially atherosclerotic diseases.12–20 Although results of numerous previously published studies have shown that elevated serum bilirubin concentrations provide important protection against atherosclerotic diseases,12,13,15–19 it is not clear whether higher serum bilirubin concentrations in physiological ranges work in favor of the cardiovascular system in younger subjects with no traditional cardiovascular risk factors.

Coronary flow reserve (CFR) is defined as the ratio of stimulated coronary blood flow velocity to baseline (resting). CFR measurement is used to assess epicardial coronary arteries and to examine the integrity of coronary microvascular circulation. In recent years, transthoracic second harmonic Doppler echocardiographic examination of CFR in the middle to distal portion of the left anterior descending coronary artery (LAD) has become very popular, and in several studies its feasibility has been validated in evaluating CFR in the middle to distal portion of the LAD.21,22 In a recently published study, Britten et al23 suggested that CFR in normal to mildly diseased arteries is an independent predictor for long-term prognosis of atherosclerosis within the next decade.
In this study, we investigated the effects of the high, intermediate, and low serum bilirubin concentrations in physiological ranges on coronary flow velocity and CFR using transthoracic second harmonic Doppler echocardiography.

**Materials and Methods**

**Study Population**

For this study, healthy subjects were selected from our hospital staff and/or healthy volunteers. Inclusion criteria were to be 18 to 45 years of age, being free of coronary risk factors, and for women to be on regular menstrual cycle. Exclusion criteria were having any systemic disease that could cause high bilirubin concentrations (eg, hemolytic and hepatic diseases) or any disease that could cause CFR impairment (eg, hypertension, diabetes mellitus, and family history for coronary artery disease (CAD) in first-degree male relatives younger than 55 years and in first-degree female relatives younger than 65 years), and morbid obesity (body mass index >35 kg/m²). Subjects using any vasoactive drug and those with changes of ST segment or T wave specific for myocardial ischemia, Q wave, and incidental left bundle branch block on ECG were excluded.

**Study Design**

Subjects who fulfilled all the inclusion and exclusion criteria (530 subjects) were consecutively registered for the study. At least 3 determinations of total serum bilirubin concentrations were performed in each subject within the last 3 months and averaged. A complete physical examination was performed. Peripheral arterial pulses and carotid bruits were searched for in particular, and sitting blood pressure was recorded. Each subject was questioned for smoking and alcohol consumption, and again for major cardiovascular risk factors. Blood glucose, total cholesterol, high-density lipoprotein (HDL) and LDL cholesterol, triglyceride, and transaminase enzyme levels in at least 12-hour fasting states were determined. Plasma high-sensitivity C-reactive protein levels were measured. Plasma high-sensitivity C-reactive protein levels were measured by a highly sensitive sandwich enzyme-linked immunosorbent assay technique. Serum bilirubin concentrations were determined by the colorimetric method on an Aeroset System (Abbott Laboratories, Abbott Park, Ill).

Because of the concern that smoking may impair CFR and confounds the results, we excluded current and recent smokers (78 subjects) from the study. Additionally, 7 subjects with slightly elevated transaminase levels (AST >40, and ALT >42 IU/mL), who had intermediate total bilirubin levels, were excluded from the study. Four subjects were excluded because of slightly elevated serum glucose levels (serum glucose >105 mg/dL). Also excluded were 1 subject because of left ventricular hypertrophy (left ventricular mass index ≥125 g/m² for men and 110 g/m² for women) and 4 subjects because of left ventricular diastolic dysfunction (mitral E/A ratio <1).

From the remaining study population (436 subjects), 3 groups of subjects were selected on the base of their total bilirubin levels; the subjects with serum total bilirubin level in the one-third upper part of normal range, who were selected in descending order of total bilirubin value from the highest, were included in the high bilirubin group (52 subjects; 23 men and 29 women, serum total bilirubin 1.43±0.33 mg/dL, mean age 35.9±7.3 years), and the subjects with serum total bilirubin level in the one-third lower part of the physiological range, who were selected in ascending order of total bilirubin value from the lowest (53 subjects; 24 men and 29 women, serum total bilirubin 0.37±0.08 mg/dL, mean age 37.6±6.6) were included in the low bilirubin group. From the remaining 331 subjects who had intermediate serum total bilirubin level, 55 subjects (27 men and 28 women, serum total bilirubin 0.69±0.11 mg/dL, mean age 36.4±6.8 years) were randomly selected for the intermediate bilirubin group.

**Coronary Flow Reserve Measurement**

Within the 3 groups, the 160 subjects underwent transthoracic echocardiographic examination including transthoracic CFR measurement. After at least 12 hours of fasting, the subjects were examined in the left lateral position. Transthoracic second harmonic Doppler echocardiography examination was performed on each subject using an Acuson Sequoia C256® Echocardiography System (Acuson Corp, Mountain View, Calif) equipped with a high-resolution transducer with second harmonic capability (5V2c). Visualization of the distal LAD was performed using a modified, foreshortened, 2-chamber view obtained by sliding the transducer on the upper part and medially, from an apical 2-chamber view, to reach the best alignment to the interventricular sulcus. Subsequently, coronary flow in the distal LAD was examined by color Doppler flow mapping over the epicardial part of the anterior wall, with the color Doppler velocity range set in the range of 8.9 to 24.0 cm/second. The color gain was adjusted to provide optimal images. The acoustic window was around the midclavicular line, in the fourth and fifth intercostal spaces, with the subject in the left lateral decubitus position. The left ventricle was imaged on the long-axis cross-section, and the ultrasound beam was then inclined laterally. Next, coronary blood flow in the LAD (middle to distal) was searched by color Doppler flow mapping. All subjects had Doppler recordings of the LAD with a dipyridamole infusion at a rate of 0.84 mg/kg over 6 minutes. All subjects had continuous heart rate and electrocardiographic monitoring as well as blood pressure recording at baseline, during dipyridamole infusion, and at recovery. Echocardiographic images were recorded on VHS videotapes. Two experienced echocardiographers who had been blinded to the clinical data analyzed the recordings. By placing the sample volume on the color signal, spectral Doppler of the LAD showed the characteristic biphasic flow pattern with larger diastolic and smaller systolic components (Figure 1). Coronary diastolic peak velocities were measured at baseline and after dipyridamole (0.84 mg/kg over 6 minutes) by averaging the highest 3 Doppler signals for each measurement. CFR was defined as the ratio of hyperemic to baseline diastolic peak velocities. CFR ≥2.0 was considered normal. CF measurement was achieved in all subjects. To test the reproducibility of CFR measurement, in 20 subjects the measurement was repeated 2 days later. Intra observer intraclass correlation coefficient for CFR measurement was 0.955.

**Left Ventricular Mass Determination**

Left ventricular mass was calculated from M-Mode records taken on parasternal long-axis images according to Devereux’s formula.

**Statistical Analyses**

Statistical analyses were performed using SPSS 9.0 (SPSS for windows 9.0, Chicago, Ill). Numeric values are expressed as mean±SD. One-way ANOVA with posthoc Tukey test was used to compare the data of the 3 groups. The correlation between numeric variables was performed with Pearson’s bivariate correlation test when appropriate. Linear regression analysis was performed to search the independent associations of CFR. P<0.05 was considered significant.

**Results**

Demographic features of the subjects and comparison of the 3 groups regarding cardiovascular risk factors are shown in Table 1. There was no significant difference among the 3 groups regarding age, sex, body mass index, family history of coronary heart disease, blood pressure, liver enzyme levels, serum concentration of fasting blood glucose, total cholesterol, triglyceride, HDL cholesterol, and LDL cholesterol. No subject had diabetes mellitus or hypertension, or hepatic, renal, hemolytic, and/or any other systemic disease. hsCRP levels were significantly lower in subjects with high bilirubin concentrations than those in both intermediate and low
bilirubin groups (1.4 ± 1.0, 2.0 ± 1.7, 3.0 ± 1.9 mg/L, respectively; \( P<0.001 \)) (Table 1).

CFR values were significantly higher in subjects with high bilirubin concentrations than those in the intermediate and the low bilirubin groups (3.19 ± 0.73; 2.75 ± 0.42; 2.56 ± 0.52, respectively; \( P=0.0001 \)) (Figure 2). Hyperemic mean peak flow velocity (hmPFV) were significantly greater in subjects with high serum bilirubin concentrations than those were in subjects with intermediate and low serum bilirubin concentrations (73.12 ± 17.36; 62.69 ± 12.49, \( P=0.001 \); 61.57 ± 13.23 cm/s, \( P<0.0001 \), respectively); however, no significant difference was found among the 3 groups regarding baseline mean peak flow velocity (23.02 ± 3.26, 22.89 ± 4.14, 24.28 ± 4.20 cm/s, respectively) (Table 2). Although CFR values of the intermediate bilirubin group were higher than those of the low bilirubin group, this difference did not reach statistical significance (Table 2). Both serum direct and indirect bilirubin levels showed the same favorable effects on CFR.

We found no significant difference among the 3 groups with respect to echocardiographic measurements including left ventricular mass index and systolic and diastolic functions (Table 2).

Plasma total bilirubin concentrations significantly correlated with hsCRP and CFR, but not with other study variables (Table 3). Among the study variables, CFR was found to be correlated with bilirubin levels, age, LDL cholesterol, mitral E velocity, and mitral E/A ratio (Table 4).

In a linear regression model when CFR was taken as dependent and other variables including age, sex, body mass index, blood pressure, lipids, and hsCRP as independent, we found that total bilirubin level has an independent association with CFR (\( \beta=0.432, P<0.0001 \)).

There was a significant correlation between plasma hsCRP and total bilirubin concentration (\( r=-0.333, P<0.0001 \). hsCRP level significantly correlated with CFR and hmPFV (\( r=-0.311, P<0.0001 \); \( r=-0.382, P<0.0001 \)), but not with baseline mean peak flow velocity (\( r=0.143, P=0.07 \)).

Intraclass correlation coefficient for bilirubin values within 3 months was found 0.82.

Discussion

Schwertner et al\(^{12}\) first reported a significant inverse correlation between bilirubin concentration and the prevalence of CAD. Additionally, they reported that lower serum bilirubin concentrations are correlated with the presence of ischemic heart disease. Confirming these results, another study demonstrated an inverse relationship between the presence of CAD and circulating total bilirubin concentration.\(^{15}\)

So far, several antioxidants have been reported to protect the cardiovascular system against the adverse effects of free radicals, some of which are mainly intravascular including bilirubin (especially unconjugated bilirubin, the predominant bile pigment in the intravascular compartment).\(^{6}\) Bilirubin is an important bile pigment with prominent cytoprotective activity against oxidative stress because of its potent antioxidant properties.\(^{12-15,26-30}\) Besides being an antioxidant, bilirubin also has anticomplement properties that protect against inflammation.\(^{31}\)

To explain plausible mechanisms of bilirubin action in preventing atherosclerosis, several mechanisms have been suggested to play a potential role in the atherogenic and cardioprotective effects of bilirubin. The most popular one is bilirubin-mediated inhibition of lipid oxidation. Lipoproteins, particularly LDL cholesterol, are highly susceptible to oxida-
Coronary flow reserve was significantly greater in subjects with high bilirubin group than that was in the other 2 groups. CFR indicates coronary flow reserve. *P<0.0001 vs low bilirubin group; †P<0.0001 vs low and intermediate bilirubin group; ‡P<0.001 vs low bilirubin group.

Figure 2. Coronary flow reserve was significantly greater in subjects with high bilirubin group than that was in the other 2 groups. CFR indicates coronary flow reserve. *P<0.0001 vs low bilirubin group.
are in line with the observations of Schwertner et al. They suggested that increased physiological concentrations of plasma bilirubin may reduce atherogenic risk.

Although it is well known that there is a prominent relationship between CAD and serum bilirubin concentrations, so far to our knowledge there has been no study published investigating the effects of serum bilirubin concentrations on normal coronary arteries in subjects with no risk factors of coronary atherosclerosis. To our knowledge, this is the first study to investigate possible effects of serum bilirubin concentration on CFR. In previous studies, presence of concomitant liver disease was not excluded in patients with hyperbilirubinemia. However, in our study, we excluded subjects with liver disease or with elevated transaminase levels to compose a totally healthy study population.

Previous studies have shown that plasma bilirubin concentrations are correlated inversely with several risk factors for CAD such as smoking, diabetes, and obesity, and correlated directly with HDL cholesterol. In our study, we excluded subjects with known risk factors for CAD to investigate the possible effects of bilirubin by itself on CFR and coronary microvascular functions; however, we could not demonstrate a direct correlation between bilirubin concentration and HDL cholesterol.

In our study, absence of a significant correlation between bilirubin concentration and HDL cholesterol might have been caused by the fact that we included subjects with bilirubin concentrations in physiological ranges. Our findings are in line with the observations of Schwertner et al. They observed the same inverse correlation between bilirubin concentration and presence of CAD, after adjustment for known CAD risk factors including age, total cholesterol, HDL cholesterol, smoking, and systolic blood pressure. It is also possible that the beneficial effect of hyperbilirubinemia on preventing coronary microvascular dysfunction and CFR impairment may be additive or synergistic to that of HDL cholesterol.

Another plausible mechanism of bilirubin action in preventing atherosclerosis might be an involvement of bilirubin in immune reactions. Involvement in inflammatory processes has also been documented. Biliverdin and bilirubin inhibit complement-dependent reactions in vitro. Biliverdin administration also inhibits anaphylactic shock produced by a single intravenous injection into guinea pigs of Forssman antibody, which interacts with Forssman antigen in their tissues. On the basis of these findings, it is possible that bile pigments are endogenous tissue protectors by virtue of their anticomplement activity. Confirming these findings, we found a significant inverse correlation between serum bilirubin concentration and hsCRP levels. According to our results, coronary flow measurements and CFR independently correlate only with serum bilirubin concentrations and age. These findings suggest that bilirubin, by preventing inflammation, might indirectly act by preventing coronary microvascular dysfunction and CFR impairment.

In addition to its antioxidative properties, bilirubin has been suggested to have cytoprotective properties through its influence on protein kinase C. In vitro, protein kinase C increases the scavenger–receptor expression in smooth muscle cells and therefore contributes to the formation of smooth muscle foam cells. The findings of Mietus-Snyder et al have been confirmed by our results that hyperbilirubinemia can improve endothelium-independent vasodilator functions of the coronary vasculature. In a recent study, Britten et al emphasized the prognostic importance of CFR with respect to atherosclerosis in subjects with normal coronary arteries or mildly diseased coronary arteries. Although Britten et al used coronary Doppler to measure CFR, validation of transthoracic Doppler echocardiographic measurement has been tested and approved by previous studies.

It has been demonstrated previously that left ventricular hypertrophy and left ventricular diastolic dysfunction can cause CFR impairment and/or coronary microvascular dysfunction. To prevent the confounding effects of left ventricular hypertrophy and left ventricular diastolic dysfunction on the study results, we excluded subjects with left ventricular hypertrophy (left ventricular mass index ≥125 g/m² for men and 110 g/m² for women) and subjects with left ventricular diastolic dysfunction (mitral E<A on pulsed-wave Doppler spectrum). However, we found that CFR significantly and positively correlated with mitral E velocity and mitral E/A ratio.

In the current study, we found no significant difference among HDL cholesterol levels of the 3 groups. This is in contrast to the findings of Vitek et al in their study of patients with Gilbert syndrome, in which their results might have been caused by different biochemical mechanisms involved in the pathogenesis of Gilbert syndrome. In the meta-analysis of Novotny and Vitek, a serum bilirubin

### TABLE 4. Correlations of CFR With Study Variables and Findings

<table>
<thead>
<tr>
<th>Variable</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>−0.234</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>−0.119</td>
<td>0.13</td>
</tr>
<tr>
<td>Total chol, mg/dL</td>
<td>−0.093</td>
<td>0.24</td>
</tr>
<tr>
<td>HDL chol, mg/dL</td>
<td>0.111</td>
<td>0.16</td>
</tr>
<tr>
<td>LDL chol, mg/dL</td>
<td>−0.223</td>
<td>0.005</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>−0.181</td>
<td>0.02</td>
</tr>
<tr>
<td>Baseline heart rate, bpm</td>
<td>−0.080</td>
<td>0.30</td>
</tr>
<tr>
<td>Baseline SBP, mm Hg</td>
<td>−0.082</td>
<td>0.30</td>
</tr>
<tr>
<td>Baseline DBP, mm Hg</td>
<td>−0.057</td>
<td>0.54</td>
</tr>
<tr>
<td>Peak SBP, mm Hg</td>
<td>−0.055</td>
<td>0.47</td>
</tr>
<tr>
<td>Peak DBP, mm Hg</td>
<td>−0.073</td>
<td>0.40</td>
</tr>
<tr>
<td>Peak heart rate, bpm</td>
<td>0.280</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hgb, g/dL</td>
<td>−0.090</td>
<td>0.57</td>
</tr>
<tr>
<td>LVMI, g/m²</td>
<td>−0.112</td>
<td>0.19</td>
</tr>
<tr>
<td>Mitral E max, cm/s</td>
<td>0.315</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mitral A max, cm/s</td>
<td>−0.022</td>
<td>0.80</td>
</tr>
<tr>
<td>Mitral E/A</td>
<td>0.216</td>
<td>0.01</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>−0.311</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

See previous Tables for abbreviations.

oxidized LDL in the development of atherosclerosis and the ability of bilirubin to serve as a potent lipid chain-breaking antioxidant under physiological conditions, it has been suggested that increased physiological concentrations of plasma bilirubin may reduce atherogenic risk.

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In the current study, we found no significant difference among HDL cholesterol levels of the 3 groups. This is in contrast to the findings of Vitek et al in their study of patients with Gilbert syndrome, in which their results might have been caused by different biochemical mechanisms involved in the pathogenesis of Gilbert syndrome. In the meta-analysis of Novotny and Vitek, a serum bilirubin
concentration of 10 μmol/L is an apparent cutoff for discrimination of cardiovascular risk.39

In conclusion, elevated concentrations of bilirubin may indeed serve as a protective factor in development of CFR impairment, coronary microvascular dysfunction, and possible development of coronary atherosclerosis. Considering our results, we might conclude that bilirubin shows these beneficial effects independently from the known coronary risk factors including HDL cholesterol. The evidence presented here confirms that serum bilirubin concentrations in the upper portion of the reference interval for a young adult population provide protection against coronary microvascular dysfunction and CFR impairment improving endothelium-independent coronary vasodilator function.

Study Limitations

In this study, we have excluded subjects with confounding factors, which are commonly encountered in normal population, such as hypertension, left ventricular hypertrophy, diabetes mellitus, morbid obesity, and current smoking for CFR to investigate the independent effects of bilirubin on CFR. Therefore, the study does not provide information about bilirubin effects on CFR in patients with risk factors for coronary heart disease, and our results cannot be applied to overall population.

References


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