

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

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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 versus 9.4 ± 2.7 $\mu\text{mol/L}$; $P < 0.001$). Serum concentrations of malondialdehyde-modified low-density lipoprotein 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 versus 72.5 ± 21.8 U/L, $P = 0.034$; 7.8 ± 2.4 versus 10.4 ± 3.2 ng/mg creatinine, $P = 0.001$, respectively). Flow-mediated vasodilation was greater in patients with Gilbert syndrome than in normal control subjects ($7.2 \pm 2.2\%$ versus $5.9 \pm 1.7\%$; $P < 0.001$). Vascular responses to nitroglycerine were not significantly different between the 2 groups. Flow-mediated vasodilation correlated with serum concentration of bilirubin ($r = 0.44$, $P < 0.001$), malondialdehyde-modified low-density lipoprotein ($r = -0.25$, $P = 0.01$), 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 malondialdehyde-modified low-density lipoprotein ($r = -0.20$, $P = 0.04$) 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.

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Key Words: bilirubin ■ endothelial function ■ oxidative stress ■ peripheral vascular diseases ■ Gilbert syndrome

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

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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 al¹⁵ showed

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that oxidative stress markers were decreased in patients with diabetes mellitus who had Gilbert syndrome compared with those in patients with diabetes mellitus who did not have Gilbert syndrome. Therefore, patients with Gilbert syndrome are ideal models for determining how endothelium-dependent vasodilation is affected by a 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 11 922 subjects of the databases of the Rho-Associated Kinase Study Registry and Flow-Mediated Dilation Japan Registry. Gilbert syndrome was diagnosed by the following criteria: mild unconjugated hyperbilirubinemia (1.2–6.0 mg/dL [20–103 $\mu\text{mol/L}$]), normal values of hepatic biochemical tests, absence of liver disease or overt hemolysis, UDP-glucuronosyltransferase 1 activity of <35% of normal, and results of real-time polymerase chain reaction 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 $\mu\text{mol/L}$). None of the subjects had a history of cardiovascular or cerebrovascular disease or 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 to 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 with the use of 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. The measurement %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 (πr^2). Reactive hyperemia was calculated as the maximum percent increase in flow after cuff deflation compared with baseline flow.

The response to nitroglycerine was used for assessment of endothelium-independent vasodilation. After baseline rest image for 30 seconds was acquired, 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. The measurement %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 (LDL) cholesterol, triglycerides, creatinine, glucose, and electrolytes. The serum concentrations of malondialdehyde-modified LDL (MDA-LDL) were assayed by enzyme-linked immunosorbent assay (anti-MDA-LDL antibody, SRL Co, Atsugi, Japan). Serum concentrations of high-sensitivity C-reactive protein were measured by a high-sensitivity nephelometry assay with the use of a C-reactive protein kit (Dade Behring, Deerfield, IL). Serum concentrations of interleukin-6 were measured by a high-sensitivity enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The urinary concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) were assayed by enzyme-linked immunosorbent assay with the use of 8-OHdG kits (Nihon Yushi Co, Tokyo, Japan).

Statistical Analysis

Results are presented as mean \pm 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 to 1 control). Paired *t* test was used for comparison of mean values of continuous variables between the 2 groups. Correlations between continuous variables were estimated with the use of Spearman correlation coefficients. The data were processed with the use of 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 2 groups.

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

Characteristic	Control Subjects (n=108)	Patients With Gilbert Syndrome (n=108)	P
Age, y	23±5	23±5	0.98
Body mass index, kg/m ²	22.5±4.3	22.9±4.3	0.85
Systolic blood pressure, mm Hg	112.2±9.4	111.6±8.8	0.72
Diastolic blood pressure, mm Hg	72.1±8.2	71.4±7.9	0.69
Heart rate, bpm	66.4±9.6	68.4±8.7	0.61
Total cholesterol, mmol/L	4.51±0.98	4.34±1.12	0.23
Triglycerides, mmol/L	1.33±0.69	1.29±0.71	0.21
HDL cholesterol, mmol/L	1.38±0.48	1.46±0.43	0.26
LDL cholesterol, mmol/L	2.26±0.77	2.41±0.58	0.73
Glucose, mmol/L	4.8±0.5	4.8±0.6	0.92
Bilirubin, μmol/L	9.4±2.7	29.2±11.6	<0.001
Interleukin-6, ng/L	1.1±2.1	1.0±1.9	0.22
High-sensitivity CRP, mg/L	0.58±0.31	0.53±0.29	0.18
MDA-LDL, U/L	72.5±21.8	61.8±24.5	0.032
Urinary excretion of 8-OHdG, ng/mg creatinine	10.4±3.2	7.8±2.4	0.001

Values are mean±SD. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; MDA-LDL, malondialdehyde-modified LDL; and 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

Vascular Function in Gilbert Syndrome

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

Relationships Between Bilirubin, Oxidative Stress Markers, and FMD

FMD was 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

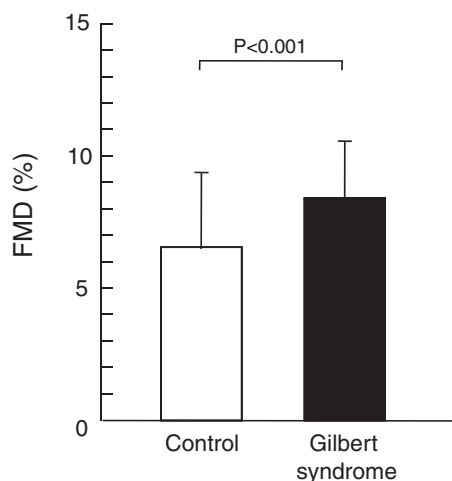


Figure 1. Flow-mediated vasodilation (FMD) to hyperemic flow in control subjects and patients with Gilbert syndrome.

Table 2. Vascular Function of Patients With Gilbert Syndrome and Control Subjects

Vascular Function	Control Subjects (n=108)	Patients With Gilbert Syndrome (n=108)	P
Brachial artery diameter, mm	3.78±0.42	3.82±0.45	0.78
Increased hyperemic blood flow, %	378±214	345±245	0.63
Nitroglycerine-induced vasodilation, %	12.0±2.3	11.8±3.2	0.45

Values are mean±SD.

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 ($r=-0.20$, $P=0.04$) (Fig-

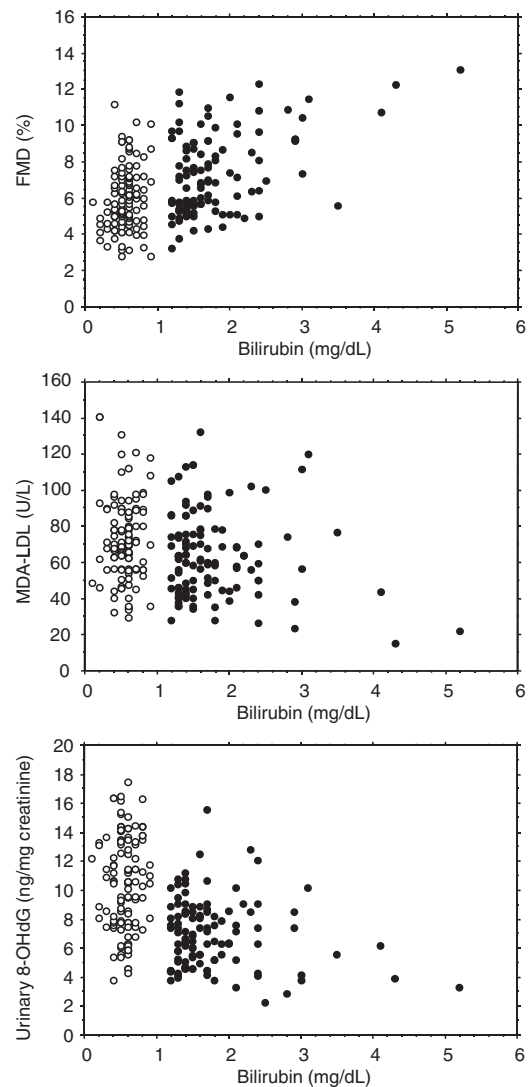


Figure 2. Scatterplots show significant correlations between serum concentration of bilirubin and flow-mediated vasodilation (FMD) ($r=0.44$, $P<0.001$, top), serum concentration of malondialdehyde-modified low-density lipoprotein (MDA-LDL) ($r=-0.20$, $P=0.04$, middle), and urinary excretion of 8-hydroxy-2'-deoxyguanosine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

ure 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 2 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 normal conditions 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 normal conditions, 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.^{4,5} Both 8-OHdG and MDA-LDL have been used as indices of oxidative stress.^{22–24} 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.²² Measurement of MDA-LDL has been proposed as the biological signature of clinical *in vivo* LDL oxidation.^{6,23} 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 an 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 humans.

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.^{25,26} In 1987, Stocker et al¹¹

for the first time demonstrated that bilirubin scavenged peroxy 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.^{11–14} It is well known that the major source of ROS in the vasculature is an NADPH oxidase activation that is induced by various stimuli.^{27,28} We have reported that endothelial function is restored by inhibition of angiotensin II-induced NADPH oxidase activity in a clinical setting.⁶ Interestingly, bilirubin inhibits NADPH oxidase activity *in vitro* and *in vivo*,^{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.^{2,3} Clinical studies have shown that serum concentrations of bilirubin inversely correlate with risk of cardiovascular diseases and peripheral arterial disease.^{31–33} In addition, the prevalence of ischemic heart disease in patients with Gilbert syndrome was only 2%, which is much lower than the prevalence of 12% in the general population.¹⁸ These findings suggest that bilirubin is a potent endogenous antioxidant and has beneficial effects on inhibition of the development of atherosclerosis, probably because of its antioxidative ability.

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.³⁴ However, in the present study, there were no significant differences in serum levels of interleukin-6 and high-sensitivity C-reactive protein 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 a 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, the existence of variability in the values of FMD and nitroglycerine-induced vasodilation may be due to differences in the methods for measurement of FMD and nitroglycerine-induced vasodilation (eg, differences in testing modality, position of cuff placement, timing of measurement of vasodilation, and skill of the observer).

It is known that the human immunodeficiency virus protease inhibitor atazanavir increases bilirubin levels through an inhibition of UDP-glucuronosyltransferase 1 activity.³⁵ Dekker et al³⁶ 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, Dubé et al³⁷ 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 have been postulated, including differences in subject selection (subjects with diabetes mellitus versus healthy subjects), differences in the increase in average bilirubin levels induced by atazanavir (3.8 versus 1.2

mg/dL), differences in the dose of atazanavir used (600 versus 400 mg/d), differences in treatment period (3 days versus 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 be aware of the existence of Gilbert syndrome because 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 normal conditions in subjects without cardiovascular risk factors.

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Disclosures

None.

References

- Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med*. 1999; 340:115–126.
- Leopold JA, Loscalzo J. Oxidative enzymopathies and vascular disease. *Arterioscler Thromb Vasc Biol*. 2005;25:1332–1340.
- Förstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med*. 2008;5:338–349.
- Drexler H, Horning B. Endothelial dysfunction in human disease. *J Mol Cell Cardiol*. 1999;31:51–60.
- Heitzer T, Schlinzig T, Krohn K, Meinertz T, Münzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation*. 2001;104:2673–2678.
- Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K. Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med*. 2002;346:1954–1962.
- Gilbert A, Lereboullet P. La cholémie simple familiale. *Semaine Med*. 1901;21:241–243.
- Owens D, Evans J. Population studies on Gilbert's syndrome. *J Med Genet*. 1975;12:152–156.
- Sieg A, Arab L, Schlierf G, Stiehl A, Kommerell B. Prevalence of Gilbert's syndrome in Germany. *Dtsch Med Wochenschr*. 1987;112: 1206–1208.
- Kapitulnik J. Bilirubin: an endogenous product of heme degradation with both cytotoxic and cytoprotective properties. *Mol Pharmacol*. 2004;66: 773–779.
- Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science*. 1987;235:1043–1046.
- Doré S, Takahashi M, Ferris CD, Zakhary R, Hester LD, Guastella D, Snyder SH. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proc Natl Acad Sci U S A*. 1999;96:2445–2450.
- Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ, Motterlini R. Heme oxygenase-1-derived bilirubin ameliorates postschemic myocardial dysfunction. *Am J Physiol*. 2000;278:H643–H651.
- Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. *Pharmacol Rev*. 2008;60:79–127.
- 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.
- Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, Oostra BA, Lindhout D, Tytgat GN, Jansen PL, Oude Elferink RP, Chowdhury NR. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med*. 1995; 333:1171–1175.
- Lin JP, O'Donnell CJ, Schwaiger JP, Cupples LA, Lingenhel A, Hunt SC, Yang S, Kronenberg F. Association between the UGT1A1*28 allele, bilirubin levels, and coronary heart disease in the Framingham Heart Study. *Circulation*. 2006;114:476–481.
- Vítek L, Jirsa M, Brodanová M, Kalab M, Marecek Z, Danzig V, Novotný L, Kotal P. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. *Atherosclerosis*. 2002;160: 449–456.
- Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation*. 1998;97:2222–2229.
- Dijhorst-Oei LT, Stores ES, Koomans HA, Rabelink TJ. Acute simultaneous stimulation of nitric oxide and oxygen radicals by angiotensin II in humans in vivo. *J Cardiovasc Pharmacol*. 1999;33:420–424.
- Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*. 2000;87:840–844.
- Nakae D, Kobayashi Y, Akai H, Andoh N, Satoh H, Ohashi K, Tsutsumi M, Konishi Y. Involvement of 8-hydroxyguanine formation in the initial rat liver carcinogenesis by low dose levels of N-nitrosodiethylamine. *Cancer Res*. 1997;57:1281–1287.
- Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation*. 1998;98: 1487–1494.
- Miyazaki T, Shimada K, Sato O, Kotani K, Kume A, Sumiyoshi K, Sato Y, Ohmura H, Watanabe Y, Mokuno H, Daida H. Circulating malondialdehyde-modified LDL and atherogenic lipoprotein profiles measured by nuclear magnetic resonance spectroscopy in patients with coronary artery disease. *Atherosclerosis*. 2005;179:139–145.
- Ryter SW, Choi AM. Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy. *Am J Respir Cell Mol Biol*. 2009;41:251–260.
- Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci U S A*. 2002;99: 16093–16098.
- Romero JC, Reckelhoff JF. Role of angiotensin and oxidative stress in essential hypertension. *Hypertension*. 1999;34:943–949.
- Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griending KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. *J Clin Invest*. 1996;97:1916–1923.
- 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.
- Lanone S, Bloc S, Foresti R, Almolki A, Taillé C, Callebert J, Conti M, Goven D, Aubier M, Dureuil B, El-Benna J, Motterlini R, Boczkowski J. Bilirubin decreases NOS2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. *FASEB J*. 2005;19:1890–1892.
- Schwertner HA, Jackson WG, Tolan G. Association of low serum concentration of bilirubin with increased risk of coronary artery disease. *Clin Chem*. 1994;40:18–23.
- 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.
- Perlstein TS, Pande RL, Beckman JA, Creager MA. Serum total bilirubin level and prevalent lower-extremity peripheral arterial disease: National Health and Nutrition Examination Survey (NHANES) 1999 to 2004. *Arterioscler Thromb Vasc Biol*. 2008;28:166–172.
- Kawamura K, Ishikawa K, Wada Y, Kimura S, Matsumoto H, Kohro T, Itabe H, Kodama T, Maruyama Y. Bilirubin from heme oxygenase-1

- attenuates vascular endothelial activation and dysfunction. *Arterioscler Thromb Vasc Biol.* 2005;25:155–160.
35. Croom KF, Dhillon S, Keam SJ. Atazanavir: a review of its use in the management of HIV-1 infection. *Drugs.* 2009;69:1107–1140.
36. Dekker D, Dorresteijn MJ, Pijnenburg M, Heemskerk S, Rasing-Hoogveld A, Burger DM, Wagener FA, Smits P. The bilirubin-increasing drug atazanavir improves endothelial function in patients with type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol.* 2011; 31:458–463.
37. Dubé MP, Shen C, Greenwald M, Mather KJ. No impairment of endothelial function or insulin sensitivity with 4 weeks of the HIV protease inhibitors atazanavir or lopinavir-ritonavir in healthy subjects without HIV infection: a placebo-controlled trial. *Clin Infect Dis.* 2008;47:567–574.

CLINICAL PERSPECTIVE

A balance between ambient levels of superoxide and nitric oxide release plays a critical role in the maintenance of normal endothelial function. Patients with Gilbert syndrome have mild unconjugated nonhemolytic hyperbilirubinemia, and the incidence of Gilbert syndrome in the general population is 5% to 10%. Gilbert syndrome is an ideal model for determining how endothelium-dependent vasodilation is affected by bilirubin-induced decrease in oxidative stress. In the present study, we found that serum concentrations of malondialdehyde-modified low-density lipoprotein and urinary excretion of 8-hydroxy-2'-deoxyguanosine, as indices of oxidative stress, were lower in 108 patients with Gilbert syndrome than in 108 control subjects and that flow-mediated vasodilation was greater in patients with Gilbert syndrome than in normal control subjects. Flow-mediated vasodilation correlated with serum concentration of bilirubin and with urinary excretion of 8-hydroxy-2'-deoxyguanosine in patients with Gilbert syndrome but not in control subjects. In addition, serum concentration of bilirubin correlated with malondialdehyde-modified low-density lipoprotein and 8-hydroxy-2'-deoxyguanosine in patients with Gilbert syndrome. Patients with Gilbert syndrome had low levels of oxidative stress associated with hyperbilirubinemia and enhancement of endothelium-dependent vasodilation. The mechanism by which endothelial function was augmented in patients with Gilbert syndrome may be due to the bilirubin-induced decrease in oxidative stress. 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. Bilirubin is a potent antioxidant and a mediator of endothelial function through inhibition of nitric oxide inactivity by its antioxidative effect.