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Objective—In type 2 diabetes mellitus (T2DM), oxidative stress gives rise to endothelial dysfunction. Bilirubin, a powerful endogenous antioxidant, significantly attenuates endothelial dysfunction in preclinical experiments. The Gilbert syndrome is accompanied by a mild and lifelong hyperbilirubinemia and associated with only one third of the usual cardiovascular mortality risk. The hyperbilirubinemia caused by atazanavir treatment closely resembles the Gilbert syndrome. We thus hypothesized that treatment with atazanavir would ameliorate oxidative stress and vascular inflammation and improve endothelial function in T2DM.

Methods and Results—In a double-blind, placebo-controlled crossover design, we induced a moderate hyperbilirubinemia by a 3-day atazanavir treatment in 16 subjects experiencing T2DM. On the fourth day, endothelial function was assessed by venous occlusion plethysmography. Endothelium-dependent and endothelium-independent vasodilation were assessed by intraarterial infusion of acetylcholine and nitroglycerin, respectively. Atazanavir treatment induced an increase in average bilirubin levels from 7 μmol/L (0.4 mg/dL) to 64 μmol/L (3.8 mg/dL). A significant improvement in plasma antioxidant capacity ($P<0.001$) and endothelium-dependent vasodilation ($P=0.036$) and a decrease in plasma von Willebrand factor ($P=0.052$) were observed.

Conclusion—Experimental hyperbilirubinemia is associated with a significant improvement of endothelial function in T2DM. **(*Arterioscler Thromb Vasc Biol*. 2011;31:458-463.)**

Key Words: antioxidants ■ atherosclerosis ■ endothelial function ■ reactive oxygen species ■ bilirubin ■ type 2 diabetes mellitus

For years, bilirubin has been recognized as a powerful antioxidant in the human body.[1,2] As atherosclerosis is characterized by a chronic state of low-grade inflammation[3] and oxidative stress of the vascular wall,[4] its development may be delayed by bilirubin. Preclinical data strongly support this hypothesis. Several in vitro experiments for example have demonstrated protection against low-density lipoprotein (LDL) oxidation by physiological or only mildly elevated bilirubin levels.[5,6] Moreover, bilirubin attenuates the proinflammatory response of vascular endothelial cells to oxidized LDL and tumor necrosis factor-α.[7] Most importantly, parenteral treatment with bilirubin ameliorated the endothelium-dependent vasodilator response of thoracic aortic rings of LDL receptor knockout mice on a high-fat diet.[7] In line with these robust preclinical data, multiple observational studies have demonstrated an inverse relationship between bilirubin levels and cardiovascular disease in humans.[8,9] Striking in this respect is the fact that subjects with a mild lifelong hyperbilirubinemia due to the Gilbert syndrome carry only one third of the cardiovascular mortality risk of subjects without the syndrome.[10]

As shown in Figure 1, bilirubin is one of the effector molecules of the cytoprotective enzyme heme oxygenase.[11] Before its excretion into the bile, it is conjugated by UDP glucuronosyl transferase 1A1 (UGT1A1). In the Gilbert syndrome, the conjugation and thus excretion of bilirubin is hampered as a result of an inactivating TA-repeat polymorphism in the promoter region of the gene coding for UGT1A1.[12] As reported recently, this polymorphism contributes substantially to the variability in bilirubin levels.[13] Not surprisingly, UGT1A1 has been suggested as an interesting drug target for the prevention of cardiovascular disease.[14] Atazanavir is an HIV-1 protease inhibitor licensed for the treatment of HIV infections and is known to inhibit UGT1A1 activity.[15] As such, treatment with atazanavir closely resembles the Gilbert syndrome.

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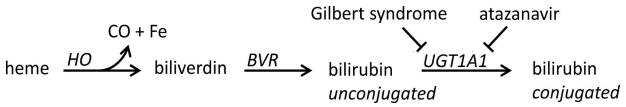

Figure 1. Bilirubin is one of the effector molecules of heme oxygenase (HO), which breaks down heme into carbon monoxide (CO), iron (Fe) and biliverdin. Biliverdin is transformed to bilirubin by biliverdin reductase (BVR). Bilirubin is conjugated by UGT1A1. Like the Gilbert syndrome, atazanavir attenuates UGT1A1 activity.

Subjects experiencing type 2 diabetes mellitus (T2DM) are particularly prone to the detrimental consequences of cardiovascular disease. Endothelial dysfunction can be demonstrated even early in the disease and is thought to be crucial in the development of atherosclerosis.[16] As the prevalence of diabetes is increasing worldwide and is expected to double to 366 million subjects during the next 2 decades,[17] the prevention of cardiovascular disease in this population is of utmost importance. Parallel to the above-mentioned observational data, the Gilbert syndrome has been associated with a significantly lower risk of cardiovascular disease in subjects with T2DM.[18]

Despite the strong evidence derived from preclinical and observational data, human experiments exploring the beneficial effect of bilirubin on cardiovascular disease have, to the best of our knowledge, not been published so far. The aim of this double-blind, placebo-controlled crossover study was to test the hypothesis that elevation of the serum bilirubin level by experimental inhibition of UGT1A1 activity would be accompanied by an improvement of endothelial function in subjects experiencing T2DM.

Methods

Subjects with T2DM were recruited through local advertising. Individuals were not admitted to the study if they had a positive history of smoking, drug abuse, or macrovascular complications of diabetes. Subjects had to be at least 18 and no older than 70 years of age. The body mass index was allowed to range from 18 to 35 kg/m^2. All hyperglycemia treatment regimens, including diet, oral medication, and insulin therapy, were accepted. Subjects were prohibited from using vasoactive medication, aspirin, or antioxidant vitamin supplements, as these drugs could influence endothelial function. To avoid pharmacokinetic interactions with atazanavir, any use of gastric acid suppressive medication and statins was discontinued during participation starting 4 weeks before the first treatment period. Subjects were enrolled only if they accepted such treatment interruption during participation. All subjects gave written informed consent before the screening visit. Subjects with clinical evidence of cardiac or pulmonary disease and subjects with laboratory evidence of renal or hepatic abnormalities were excluded. Finally, subjects were genetically tested for the presence of the Gilbert syndrome and excluded if positive. The study protocol was approved by the local Medical Research Ethics Committee and consistent with the Declaration of Helsinki.

In a double-blind and randomized crossover study, subjects received a 3-day atazanavir treatment (Reyataz, Bristol-Myers Squibb BV, Woerden, the Netherlands) and a 3-day placebo treatment, with a washout period of at least 3 weeks in between. To amplify the level of hyperbilirubinemia, the regular dose regimen of atazanavir in HIV patients (either 400 mg once daily or 300 mg boosted with ritonavir 100 mg once daily) was modified to an alternative regimen of 300 mg twice daily to be taken with food. If reflected by the area under the curve, the exposure to atazanavir caused by this dose regimen does not exceed the exposure associated with both regular dose regimens.[19]

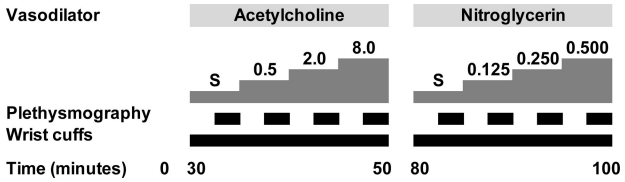

Figure 2. The vasodilator responses to acetylcholine and to nitroglycerin were consecutively assessed. Both vasodilators were administered in 3 increasing doses. Dosages were adjusted to forearm volume (depicted as μg/min per dL forearm volume). A 30-minute pause (equilibration period) was made before administration of each agent. S indicates saline.

On the fourth day of both treatment periods, forearm blood flow (FBF) was assessed by venous occlusion strain gauge plethysmography. A time schedule of these flow assessments is provided in Figure 2. All experiments were performed in the morning after an overnight fast in a temperature-controlled room (23°C), with the subjects in a supine position. If needed, dose adjustments of hypoglycemic agents were recommended during the evening and early morning before the assessments. Capillary glucose levels were monitored. The brachial artery of the nondominant arm was cannulated with a 27-gauge needle (kindly supplied by B. Braun Medical BV, Oss, the Netherlands) for intraarterial administration of saline, acetylcholine (Miochol, Thea Pharma NV, Zoetermeer, the Netherlands), and nitroglycerine (Nitropohl, Pohl-Boskamp, Hoofddorp, the Netherlands). FBF was assessed during the successive administration of 3 increasing doses of acetylcholine (0.5, 2, and 8 μg/min per dL of forearm tissue) and nitroglycerin (0.125, 0.25, and 0.5 μg/min per dL). Both series were preceded by a 30-minute pause and started with the assessment of baseline FBF during saline infusion. Each dose was administered for 5 minutes. FBF was recorded simultaneously on both the infusion and the control arm by venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, Hokanson, Inc). The upper arm cuffs were inflated using a rapid cuff inflator (Hokanson E-20, DE Hokanson, Bellevue, WA). Wrist cuffs were inflated to 220 mm Hg during each series. Immediately after completion of FBF assessments, blood pressure in the supine position was assessed with an aneroid sphygmomanometer.

Before the experiment, venous blood was drawn. Hematologic parameters were assessed using an ADVIA 120 Hemalog (Bayer Diagnostic, Tarrytown, NY), and chemical parameters, including bilirubin levels, were determined using an Aeroset (Abbott Laboratories, Abbott Park, Ill). In addition, plasma samples were stored at −80°C for determination of atazanavir plasma levels, antioxidant capacity, and biomarkers of vascular inflammation. Atazanavir plasma levels were determined using a modification of a validated high-performance liquid chromatography method with UV detection as published previously.[20] Plasma antioxidant capacity was assessed by means of the ferric reducing ability of plasma assay, according to the method of Benzie and Strain.[21] Ferric reducing ability of plasma values were obtained using a 7-point calibration curve of known amounts of Fe^{2+} and expressed in mmol Fe^{2+}/L. The concentrations of soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble intercellular adhesion molecule-1 (sICAM-1) were determined by a multiplex assay (Bio-plex cytokine assay, Bio-Rad, Hercules, Calif, at Luminex 100, Luminex Corp). The concentration of von Willebrand factor (vWF) was determined by enzyme-linked immunosorbent assay (Progen Biotechnik, Heidelberg, Germany; R&D Systems, Minneapolis, Minn). To enable the determination of bilirubin levels after deblinding of the study, 1 additional lithium heparin plasma sample was stored at −80°C in a brown Eppendorf tube.

Statistical analysis was performed using the SPSS (version 16.0) and SAS (version 8.2) software packages. The paired-samples *t* test was used to compare gaussian distributed data. For analysis of the FBF measurements, the last 5 flows of each dose were used. Before analysis, logarithmic transformation was performed to obtain a gaussian distribution. The flow data were then averaged per dose and subsequently analyzed in a mixed linear model with random factor

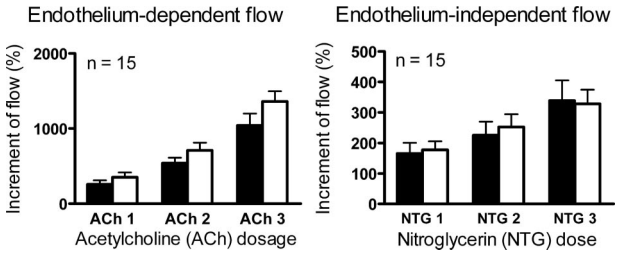

Figure 3. Increase in FBF in response to the 3 increasing dosages of acetylcholine and nitroglycerin, depicted as percentage from baseline. Error bars indicate standard errors of the mean. Black columns represent placebo treatment, and white columns represent atazanavir treatment. Statistical analysis was performed on the log-transformed data depicted in table 3.

subjects and fixed factors treatment and dose. In a post hoc analysis, the atazanavir level, the bilirubin level following atazanavir treatment, the baseline level of glycohemoglobin, and the time since diagnosis were included in the model to explore the possible impact of these factors on the degree of therapeutic response. Flow data are presented in 3 ways. Figure 3 displays the original data in terms of percentage from baseline. The log-transformed data used for analysis are presented in Table 3. The data discussed in the text are medians instead of averages of the original flow data to approximate the effect of logarithmic transformation. Statistical significance was accepted at the 95% confidence level ($P \leq 0.05$).

Results

Eighteen nonsmoking subjects with T2DM and a negative history for cardiovascular disease were recruited and gave written informed consent. Two of them were excluded during screening, one because of a genetically confirmed Gilbert syndrome and the other because of an observed tendency toward vasovagal collapse, which would have interfered with the assessment of FBF. Based on a data review following completion of the study, 1 of the 16 participating subjects was excluded because of a highly inaccurate intake of study medication, as well as an exceptionally low quality of the FBF assessments. This decision was made before the deblinding procedure. Based on post hoc analysis, inclusion of this subject would have resulted in a higher level of significance. The results presented are those of the remaining 15 subjects.

Characteristics of the study population are shown in Table 1. We examined 6 males and 9 females with an average body mass index of 28 kg/m^2. The mean history of diabetes was 5.9 years, and the average glycohemoglobin level was 6.8%. Three subjects were treated with a diet only. The other 12 subjects were treated with either monotherapy or combination therapy containing a biguanide, sulfonylurea, or insulin. Nine of 15 subjects were taking statins on inclusion and agreed with interruption during participation. As a result of the exclusion criteria, none of the subjects was using aspirin or antihypertensive medication.

Table 1. Characteristics of Study Population (n=15)

Characteristic	Value
Sex	6 men, 9 women
Age (years)	61 (6, 51 to 70)
Body mass index (kg/m^2)	28 (4, 18 to 35)
HbA1c (%)	6.8 (1.1, 5.5 to 9.8)
Treatment regimen	3 diet only
	10 metformin
	7 SU
	2 insulin
	9 statin

Data are given as mean (SD, range); frequency of treatment regimens is given as number of subjects. HbA1c indicates average glycated hemoglobin; SU, sulfonyl urea derivates.

Table 2. Laboratory Results After Atazanavir and Placebo Treatment

	Placebo	Atazanavir	*P* Value
Bilirubin (μmol/L)	7 (1)	64 (21)	<0.01
Glucose (mmol/L)	8.4 (0.6)	8.5 (0.7)	0.78
LDL (mmol/L)	3.7 (0.2)	3.6 (0.2)	0.61
FRAP (mmol Fe^{2+}/L)	1.26 (0.06)	1.66 (0.07)	<0.001
vWF (U/mL)	1.46 (0.13)	1.18 (0.12)	0.05
sVCAM-1 (pg/mL)	183 (10)	191 (8)	0.14
sICAM-1 (pg/mL)	158 (7)	153 (7)	0.37

Data are given as mean (SEM). FRAP, ferric reducing ability of plasma. Reported *P* values are the result of paired *t* tests.

Total bilirubin levels after placebo and atazanavir treatment are shown in Table 2. The average bilirubin level following placebo treatment amounted to 7 μmol/L (0.4 mg/dL). As anticipated, the 3-day course of UGT1A1 inhibition resulted in significantly elevated bilirubin levels, with an average of 64 μmol/L (3.8 mg/dL) and a range of 35 to 110 μmol/L (2.1 to 6.4 mg/dL). Because of the short term of the atazanavir treatment, significant tissue accumulation and thus clinically perceptible jaundice occurred in only 1 case. In this subject, jaundice arose on day 4 after completion of the FBF assessments.

When compared with placebo, a significant improvement of antioxidant capacity was observed following atazanavir treatment ($P<0.001$). In addition, atazanavir treatment was associated with a decrease in plasma vWF ($P=0.052$). Plasma levels of sVCAM-1 and sICAM-1 were not affected. Atazanavir treatment did not influence the plasma levels of fasting glucose and LDL cholesterol.

The results of FBF experiments are depicted in Figure 3 and Table 3. Baseline flow after placebo treatment was

Table 3. Forearm Blood Flow Data After Logarithmic Transformation

	Placebo	Atazanavir	*P* Value
Baseline	0.25 (0.04)	0.18 (0.05)	
ACh1	0.75 (0.07)	0.78 (0.07)	
ACh2	1.01 (0.06)	1.05 (0.06)	0.036
ACh3	1.25 (0.06)	1.33 (0.04)	
Baseline	0.25 (0.05)	0.21 (0.04)	
NTG1	0.64 (0.04)	0.64 (0.04)	
NTG2	0.72 (0.05)	0.73 (0.04)	0.404
NTG3	0.85 (0.04)	0.81 (0.04)	

ACh1, ACh2, and ACh3 indicate acetylcholine dosages of 0.5, 2.0, and 8.0 μg/min per dL, respectively; NTG1, NTG2, and NTG3, nitroglycerin dosage of 0.125, 0.250, and 0.500 μg/min per dL, respectively. SEM values are shown in parentheses.

comparable to baseline flow after atazanavir treatment (1.8 and 1.5 mL/min per dL tissue in the intervention arm and 1.6 and 1.7 mL/min per dL in the control arm). Neither acetylcholine infusion nor nitroglycerin infusion affected the blood flow of the contralateral forearm (data not shown).

Intraarterial infusion of acetylcholine induced an increase in FBF at all 3 doses after both placebo and atazanavir treatment. Compared with placebo, atazanavir treatment was accompanied by a significantly enhanced vasodilator response to acetylcholine. At the highest acetylcholine dose, 8 μg/min per dL, FBF amounted to median levels of 19.9 and 21.9 mL/min per dL following placebo and atazanavir treatment, respectively. Statistical analysis on the log-transformed flow data of all 3 acetylcholine dosages revealed a relative increase of 12% without relevant differences between the 3 acetylcholine dosages. The improvement of acetylcholine response was not influenced by the atazanavir level, the bilirubin level following atazanavir treatment, the glycohemoglobin level at baseline, or the duration of diabetes before inclusion (based on post hoc analysis).

Intraarterial infusion of nitroglycerin also induced a significant increase in FBF at all dosages. Contrary to acetylcholine, the extent of vasodilator response to nitroglycerin was not influenced by our intervention with atazanavir. At the highest nitroglycerin dose, 0.5 μg/min per dL, FBF amounted to median levels of 7.3 and 7.1 mL/min per dL following placebo and atazanavir treatment, respectively.

Discussion

A multitude of preclinical and observational studies have credited bilirubin with the potential to prevent cardiovascular disease. To our knowledge, this is the first study addressing the concept of experimental hyperbilirubinemia in humans. Our findings demonstrate that even a 3-day atazanavir treatment improves endothelial function in subjects with T2DM.

Endothelial dysfunction is strongly related to the development of atherosclerosis and the resulting cardiovascular risk.[22] Central in the pathogenesis of endothelial dysfunction is the decreased availability of endothelial nitric oxide (NO).[23] One of the key factors leading to limited NO availability is the increase in intracellular oxidative stress. A substantial part of the production of reactive oxygen species is supposed to stem from NADPH oxidase activity.[24]

Addressing the mechanistic importance of oxidative stress, several groups have studied the effect of antioxidant treatment strategies, among which is intraarterial infusion of ascorbate. Underscoring the fundamental role of oxidative stress, improvement of endothelial function following parenteral administration of ascorbate was observed in subjects experiencing various conditions, such as hypercholesterolemia,[25] hypertension,[26] and both insulin-independent[27] and insulin-dependent[28] diabetes mellitus. Bilirubin is a powerful endogenous antioxidant, and its clinical relevance is highly suggested by the results of preclinical and observational studies. As such, artificial elevation of the serum bilirubin level might be an attractive and long-term workable approach for the prevention of cardiovascular disease.[14,29] Our current data on the significant improvement of antioxidant capacity and endothelial function observed after a 3-day atazanavir treatment strongly support the potential of this strategy.

Biomarkers of vascular inflammation such as vWF, sVCAM-1 and sICAM-1 have been shown to be related to the risk of cardiovascular complications in T2DM.[30] In addition, bilirubin has been shown to attenuate H_2O_2-induced endothelial leukocyte rolling and adhesion in vivo.[31] The observed trend toward a decrease in serum vWF is in line with the observed improvement of endothelial function and may reflect a decrease in vascular inflammation in our subjects. The limited size of our study population and the short duration of atazanavir treatment may account for the fact that sVCAM-1 and sICAM-1 did not alter.

Several limitations should be addressed, as they may have influenced the outcome of our study. First, there are no data available on the bilirubin levels needed to obtain maximally protective antioxidant effects in vivo in humans. In our design, we opted for a short-term exposure, aiming at moderately elevated bilirubin levels. The 3-day treatment regimen resulted in a mean bilirubin level of 64 μmol/L (3.8 mg/dL). Because we did not observe a relationship between bilirubin levels and the vasodilator response to acetylcholine, this may indicate that we reached the plateau of the concentration-effect curve. Considering the relatively low bilirubin levels and the still marked cardiovascular protection observed in subjects with the Gilbert syndrome, long-term treatment regimens aiming at bilirubin levels in the subclinical range may be sufficient as well. This would favor long-term application of a lower dose of atazanavir, as our current regimen would definitely cause an unacceptable degree of jaundice during prolonged treatment. Further research is needed to address this topic.

Second, atazanavir itself may have a direct beneficial impact on endothelial function. In a previous study, 400 mg of atazanavir once daily did not influence endothelial function in healthy volunteers.[32] In our study, however, we administered a different dose. Moreover, we included patients with T2DM instead of healthy subjects. Therefore, we do not know whether our dose regimen of 300 mg twice daily might affect endothelial function in healthy subjects. Besides, bilirubin is obviously not the only substance conjugated by UGT1A1. It is likely, therefore, that the Gilbert syndrome and atazanavir treatment affect plasma levels of substances other than bilirubin. Theoretically, such substances, as well as atazanavir itself, could cause the observed improvement of vascular function ascribed to bilirubin. Nevertheless, UGT1A1 inhibition and parenteral administration of bilirubin comparably attenuated oxidative stress and hypertension in an angiotensin II–dependent animal model.[33] In our opinion, this supports a causal and dominant role of bilirubin. A similar approach with parenteral administration of bilirubin in humans may provide a definite proof of its beneficial impact on cardiovascular disease.

Finally, HIV protease inhibitors are commonly connoted for their contribution to cardiovascular complications in HIV patients.[34] In contrast to several other protease inhibitors, however, atazanavir does not affect glucose tolerance, plasma cholesterol level, or endothelial function in healthy volunteers at a dosage of 400 mg once daily.[32] Consistently, we did not

observe changes in plasma glucose or plasma cholesterol levels at a twice-daily dosage of 300 mg, nor did we find a relationship between atazanavir plasma levels and the observed improvement of endothelial function.

In contrast to the promising data on the parenteral use of ascorbate, clinical trials with orally administered exogenous antioxidants such as vitamins C and E have been generally disappointing.[35,36] Several explanations have been put forward, from the inability to obtain sufficiently elevated intracellular levels by oral dosing regimens to the inability of vitamins C and E to compete with highly reactive molecules such as peroxynitrite.[16] Notably, bilirubin is one of the most potent scavengers of reactive oxygen species in nature.[37] As our strategy evidently fortifies an endogenous and physiologically relevant antioxidant resource, it may overcome the flaws of previous treatment strategies with exogenous antioxidants. Besides, bilirubin has shown to inhibit NADPH oxidase activity in vitro.[38] Given the importance of NADPH oxidase activity in the pathogenesis of diabetes related endothelial dysfunction, this property may contribute to the beneficial effects on endothelial function observed in our diabetic subjects too.

In summary, our study is the first to address the concept of vascular protection by experimental hyperbilirubinemia in humans. Given the overwhelming preclinical and observational data on bilirubin and cardiovascular disease, it is in our opinion very likely that the improvement of endothelial function and plasma antioxidant capacity observed after atazanavir treatment should be attributed to the associated hyperbilirubinemia. Indisputable evidence on this should be provided by the use of alternative human models for experimental hyperbilirubinemia. Finally, optimally protective plasma levels have to be established. If potent at only mildly elevated bilirubin levels, long-term UGT1A1 inhibition may prove a novel pharmacological approach to prevent cardiovascular disease in T2DM.

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Disclosures

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