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SYMPOSIUM

Further evidence of altered redox status of hyperbilirubinaemic patients: role of bilirubin in Gilbert syndrome+

Krisztina Hagymási*, Ibolya Kocsis, Gabriella Lengyel, Péter Sipos, János Fehér, Anna Blázovics

2nd Department of Medicine, Semmelweis University, Budapest, Hungary

ABSTRACT Bilirubin is regarded as the most powerful endogenous antioxidant substance. It exhibits immunmodulator, inhibitor activities on kinases, yet it is clear that it can be potentially cytotoxic. Gilbert syndrome is characterised by hereditary, chronic, mild unconjugated hyperbilirubinaemia. 12 Gilbert syndrome patients and 15 healthy controls were investigated with special regard to reduction-oxidation status and free radical-antioxidant balance. Sera free SH-group concentration, H-donating ability, reducing power were measured spectrophotometric methods. Total scavenger capacity, describing free radical-antioxidant balance, was determined by a newly developed chemiluminometric method in sera, plasma and erythrocytes. Patients with Gilbert syndrome showed a significant increase of non-enzymatic antioxidant capacity. Elevated free SH-group concentration, H-donating ability and reducing power were found in mild hyperbilirubinaemia compared with control group patients. On the other hand no significant differences were detected regarding free radical-antioxidant balance in sera, plasma and erythrocytes between the groups. On the basis of these results it can be supposed that elevated bilirubin concentration, via indirect or compensatory way, strengthens non-enzymatic antioxidant capacity, without changes in antioxidant-free radical balance. That is why further investigations are needed to clarify the consequences of elevated bilirubin concentration on cell redox homeostasis
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KEY WORDS

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total scavenger capacity

Gilbert syndrome is a chronic non-hemolytic unconjugated hyperbilirubinaemia, occuring in the absence of liver disease or overt haemolysis and characterised by episodes of mild intermittent jaundice. It is the most common inherited disorder of hepatic bilirubin metabolism, occuring of 2-12 percent of the general population. Gilbert syndrome is caused by a reduction in the activity of hepatic bilirubin UDP-glucuronosyl-transferase (UGT1A1) to about 30 percent of normal. The reduction of this gene activity has been shown due to a polymorphism in the promoter region of the UGT1A1 gene, the presence of superfluous thyamin adenine repeats reduces the efficiency of transcription of this gene. The rarer, more severe and dominantly inherited forms identified to date are heterozygosity for a nonsense mutations in the coding region of the UGT1 gene (Bosma et al. 1995; Monaghan et al. 1996; Beutler et al. 1998).

UGT1A1 polymorphism is suggested that may provide a flexible polymorphism that maintain bilirubin levels in a range high enough to protect against oxidative stress, but not so high as to cause a high incidence of kernicterus (Beutler et al. 1998). Bilirubin exhibits intriguing biological activities as an antioxidant, an antimutagen and an anti-complement agent. Some authors suggest that neonatal hyperbilirubinaemia could be a transitional antioxidative mech-anism in the circulation of human neonates (Marilena 1997). Antioxidant property of bilirubin is seemed to be responsible for the reduced risk of coronary artery disease in Gilbert syndrome (Vitek et al. 2002).

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*Corresponding author. E-mail: hkriszti@bel2.sote.hu
+In memory of Professor Béla Matkovics

Materials and Methods

We studied the redox status and free radical-antioxidant balance in 12 Caucasians patients with Gilbert syndrome, ranging from 18 to 52 years (mean±SEM: 27.08±2.73 years; 11 male, 1 female), in whom the syndrome was clinically diagnosed based on a consistent mildly raised non-fasting serum total bilirubin concentration (range: 25-59 μmol/l) and on the absence of structural liver disease proved by utrasonography. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values were normal (range ALT: 11-40 U/l; AST: 15-27 U/l). Hemolysis was excluded on the basis of normal hemoglobin value and reticulocyte counts. 16 Caucasians healthy subjects were examined (age:

Table 1. Routine laboratory parameters in Gilbert syndrome and control patients.

	Total bilirubin concentration (μmol/l)	AST (U/l)	ALT (U/l)	GGT (U/l)	ALP (U/l)
Healthy controls	8.50±0.87	17.00±1.35	15.75±2.21	18.00±5.30	157.53±39.43
Patients with Gilbert syndrome	36.67±2.37	23.17±1.14	28.33±4.50	21.83±3.27	194.83±18.11

27.42±2.35 years; 8 male, 4 female) with no known history of jaundice and with normal serum total bilirubin concentration. (In our laboratory the upper limit of normal for serum total bilirubin concentration is 25 μmol/l. Normal values for AST: 10.00-37.00 U/l; ALT: 5.00-40.00 U/l; GGT: 7.00-50.00 U/l; ALP: 98.00-279.00 U/l)

The study was approved by the Regional Committee of Science and Research Ethics, Semmelweis University (permission number: TUKEB 186/1998). Informed consent was obtained from all subjects. Authors ensure that their work complies with the Declaration of Helsinki (1964).

Plasma and erythrocytes were separated with routine methods and the haemoglobin contents of samples were adjusted to 10 g/l for measurements for Haemisol reagent. The reducing power of the samples were determined at 700 nm according to the method of Oyaizu (1986), based on the chemical reaction Fe (III) → Fe(II). Increased absorbance indicated greater reducing power, which was expressed as the ascorbic acid equivalent (mmol/leqAS). The hydrogen-donating ability of the samples was estimated in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical at 517 nm on the basis of the method of Hatano et al. (1988). DPPH stable radical was found to oxidise cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines. Plasma free SH-group concentrations were measured by the Sedlak and Lindsay (1968) method. The total scavenger capacity (TSC) was determined in plasma, sera and erythrocytes by a chemiluminescence assay with Berthold Lumat 9501 manual luminometer, in H_2O_2/$OH^{\cdot}$-luminol-microperoxidase system to assess the antioxidant capacity and free radicalantioxidant balance. This luminometer is designed to determine single photons of light emitted in the course of a chemical reaction between luminol and free radicals. Intensity of the standard light of H_2O_2/$OH^{\cdot}$-luminol-microperoxidase system changes depending on the antioxidant-free radical balance in the biological sample and expressed as the percentage of the standard light (RLU%= relative Light Unit%). Higher chemiluminometric intensity indicates lower total scavenger capacity (Blázovics et al. 1999).

1,1-diphenyl-2-picrylhydrazyl, 5,5'-dithiobis-2-nitrobenzoic acid, luminol, microperoxidase were obtained from Sigma (St. Louis, USA). Haemsiol reagent was bought from Human Oltóanyag (Gödöllő, Hungary). All other reagents were purchased from Reanal (Budapest, Hungary).

One-way ANOVA statistical analysis was used to evaluate the significance between patient groups. Data are expressed as mean±SEM. $P<0.05$ was considered to indicate statistical significance.

Results

Total bilirubin concentration in sera showed a significant increase in patients with Gilbert syndrome compared to control group (36.67±2.37 vs. 8.50±0.87 μmol/l). Liver enzymes activities were slightly elevated in hyperbilirubinemic patients, however they did not exceed normal values (Table 1). Significantly elevated H-donating ability (50.68±0.97 vs. 46.11±1.10%), reducing power (1.599±0.004 vs. 1.322±0.088 nmol/leqAS) and free SH-group concentration (0.35±0.009 vs. 0.43±0.013 mmol/l) could be observed in Gilbert syndrome compared to that of control subjects (Table 2). On the other hand no significant differences were observed regarding total scavenger capacity of plasma, sera as well as erythrocytes between healthy controls and Gilbert syndrome patients (Table 3). Bilirubin concentration showed significant correlation only with reducing power (R^2=0.4080, P=0.0253). No significant relation was found in connection with free SH-group concentration (R^2=0.0012, P=0.9142), H-donating ability (R^2=0.2509, P=0.0971) and total scavenger capacity (R^2=0.0655, P=0.4217).

Table 2. Non-enzymatic antioxidant capacity of sera.

	H-donating ability (%)	Reducing power property (nmol/leqAS)	Free SH-group concentration (mmol/l)
Healthy controls	46.11±1.10	264.43±17.59	0.35±0.009
Patients with Gilbert syndrome	50.68±0.97*	319.90±8.08*	0.43±0.013*

*sign. (P<0.05) vs. control group

Table 3. Total scavenger capacity of sera, plasma and erythrocytes.

	Sera (RLU%)	Plasma (RLU%)	Erythrocytes (RLU%)
Healthy controls	20.41±1.61	1.42±0.16	54.11±4.63
Patients with Gilbert syndrome	17.59±1.94	1.44±0.24	55.94±1.76

Discussion

Bilirubin (conjugated/non-bound, non-conjugated/albuminbound) is regarded as a member of the antioxidant family, even though it is known to have toxic effects at high concentrations. Bilirubin could be an important cytoprotector for tissues which are less equipped for antioxidant defence like myocardium and nervous tissues (Marilena 1997). Along with ascorbate and urate, this bile pigment is a very important antioxidant in plasma, can act synergistically with vitamin E in protecting lipid membranes from peroxidation initiated within the lipid phase (Stocker et al.1990; Marilena 1997).

Bilirubin is a potent scavenger of singlet oxygen and peroxyl radicals with high efficiency by initial donation of a hydrogen atom of the tetrapyrrole ring (Stocker et al. 1990; Dudnik and Khrapova 1998; Minetti et al. 1998; Yesilkaya et al. 1998). Bilirubin does react with superoxide anion to some extent and serves as a substrate for peroxidases in the presence of hydrogen peroxide or organic hydroperoxides (Stocker et al. 1987). The superoxide scavenger activity similar to that of serum albumin, higher than the vitamin E analog Trolox and lower than ascorbic acid (Marilena 1997). Bilirubin participates as a scavenger of secondary oxidants formed in the oxidative process (Minetti et al. 1998). Addition of bilirubin to erythrocytes incubated with cumeneOOH (shows a similar effect to the other organic hydroperoxides and produces alkoxyl and peroxyl radicals) induced an inhibition in lipid peroxidation processes, preserving SOD activity, increasing catalase and glucose-6-phosphate dehydrogenase activity and reduced glutathione concentration (Yesilkaya et al. 1998).

Bilirubin also possess antimutagenic properties and conjugated bilirubin shows an inhibitory effect on complement dependent reactions in vitro, blocking complement cascade, especially at the C1 step (Marilena et al. 1998).

It is clear that bilirubin can be toxic to cells at higher concentrations (>30 mg/dl; Mireles et al. 1999). Bilirubin caused cytotoxicity is amplified by TNF-alfa and endotoxin. These results provide a supportive evidence that sepsis would increase the risk of tissue damage by bilirubin, the sepsis may enhance the risk of kerinicterus (Ngai and Yeung 1999). It has been recently reported that bilirubin forms a complex with Cu(II), which results in the reduction of Cu(II) to Cu(I) and this redox cycle gives rise to the formation of reactive oxygen species, particularly hydroxyl radical causing DNA breakage (Asad et al. 1999). It was deduced that bilirubin free radical initiates and promotes the pigment gallstone formation. Bilirubin free radicals were detected by electron spin resonance. The main target of bilirubin free radical is the cell membrane and membrane bound protein (Blázovics et al. 1997; Liu and Hu 2002).

Bilirubin is also a potent immunomodulator: inhibits responses of human lymphocytes, including phytohemagglutinin- induced proliferation, interleukin-2 production and antiobody dependent and independent cell-mediated cytotoxicity (Haga et al. 1996, Maines et al. 1999). This observation may explain the increased susceptibility to infection observed in hyperbilirubinemic patients (Haga et al. 1996). Bilirubin can inhibit protein kinases (cAMP-dependent, cGMP-dependent, Ca^{2+}-calmodulin-dependent Ca^{2+}-phospho-lipid dependent) by a non-competative mechanism, mod-ulating the protein phosphorilation in cellular regulation contributed to its neurotoxicity (Hansen et al. 1996). This kinases initiate and regulate various signal transduction processes including those involved in cell proliferation (Maines et al. 1999).

Earlier studies presumed that elevated bilirubin concentration in Gilbert syndrome serve as an increased antioxidant capacity (Vitek et al. 2002). The object of this study was to investigate the redox status, the free radical antioxidant balance in Gilbert syndrome patients by newly applicated spectrophotometric and newly developed luminometric methods. The mild hyperbilirubinaemia caused elevated SH-group concentration, H-donating ability and reducing power in sera. There were no significant correlations between bilirubin concentration and free SH group concentration as well as H-donating ability. On the other hand strong relation was found between bilirubinaemia and reducing power. On the basis of literature we give the following explanations for our results. Bilirubin may reduce – in consequence its antioxidant property – the utilisation of the free SH group and other antioxidant compounds and molecules, and therefore reinforce antioxidant capacity of tissues. Then again, strengthened antioxidant capacity caused by the mild hyperbilirubinaemia may be in connection with a compensatory mechanism against toxic bilirubin.

On the other hand a shift of free radical–antioxidant balance was not proved by our chemiluminometric mesaurements. We did not detect any significant differences between Gilbert syndrome and healthy control patients in sera, plasma and erythrocytes and tendency was not observed as well. On the basis of these results it can be supposed that elevated bilirubin concentration, via indirect or compensatory way, strengthens non-enzymatic antioxidant capacity, without changes in antioxidant-free radical balance. Further studies

are needed to investigate the consequences of elevated bilirubin concentration in cell redox homeostasis, on the ground its free SH-group concentration elevating, H-donating and reducing power.

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