Association of Serum Bilirubin Concentration with Risk of Coronary Artery Disease

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Background: Lipid oxidation and formation of oxygen radicals are important elements of arterial plaque formation and atherosclerosis, and are involved in the pathophysiology of coronary artery disease (CAD). Because bilirubin has antioxidant properties, it has been suggested that it may have a protective role in the atherosclerotic process.

Approach: This review examines in vitro and in vivo studies indicating that bilirubin inhibits lipid oxidation and oxygen radical formation. Experimental and epidemiological evidence is presented that suggests that bilirubin may serve as a physiological antioxidant providing protection against atherosclerosis and CAD. Special attention is focused on studies that noted an inverse relationship between plasma bilirubin concentration and cardiovascular morbidity.

Content: Serum bilirubin concentrations in the upper portion of the reference interval reportedly reduce atherogenic risk and provide protection against CAD. In contrast, serum bilirubin concentrations in the lower portion of the reference interval may be associated with increased risk of ischemic heart disease.

Summary: Taken together, the evidence presented in this review supports the concept that bilirubin, via its antioxidant potential, has antiatherogenic properties and that an inverse relationship exists between circulating bilirubin concentrations and risk of CAD.

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For many years, the bile pigment bilirubin was considered a toxic waste product formed during heme catabolism. However, more recent evidence suggests that bilirubin is a potent physiological antioxidant that may provide important protection against atherosclerosis, coronary artery disease (CAD), and inflammation. It is generally accepted that oxidative reactions are involved in the pathophysiology of these disease processes. Substantial evidence has documented that the development of CAD involves lipid oxidation and formation of oxygen radicals and that atherosclerosis and inflammation are associated with formation of oxygen and peroxyl radicals (1–3).

The antioxidant capacity of bilirubin and its ability to provide potent scavenging of peroxyl radicals have led to suggestions that mildly increased circulatory bilirubin may have a physiologic function to protect against disease processes that involve oxygen and peroxyl radicals.

This review briefly examines current evidence that bilirubin metabolism affects CAD and that bilirubin may have a protective function in suppressing atherosclerosis, CAD, and inflammation.

Heme Oxygenase: The Synthesis of Bilirubin

Heme oxygenase (HO) is the rate-limiting enzyme of bilirubin production. It is a microsomal enzyme, present in both central and peripheral tissues, that converts heme to biliverdin and CO (4). Biliverdin is subsequently reduced to bilirubin by the cytosolic enzyme biliverdin reductase (5).

At least two isoforms of HO have been identified and found to be products of different genes and to differ in their tissue expression, function, and ability to respond to stimuli (4, 6, 7). HO-1 ($M_r \cong 32\,000$) is an inducible form that is expressed at a low concentration in vascular endothelial and smooth muscle cells and is markedly induced by heme, metals, oxidative stress, inflammatory mediators, oxidized LDL, and hypoxia. A variety of experiments have suggested that HO-1 is a stress-response protein that plays an important function in cell defense mechanisms against oxidative injury. HO-1 activity is responsible for increased CO and bilirubin formation as well as iron release in pathological conditions such as cardiovascular shock, hypoxia, ischemia-reperfusion, and hypertension (4, 6–13).

Human HO-1 deficiency is associated with growth retardation, hemolytic anemia, persistent endothelial damage, iron deposition, and sensitivity to hemin-induced cell injury

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(14). In mice, HO-1 knockout is associated with growth retardation, iron deposition, and sensitivity to cell injury (15). Several experiments in which the expression of HO-1 was up-regulated by different modulators or by gene transfer suggested that HO-1 participates in defense mechanisms against agents that induce oxidative injury (12). It has also been shown that expression of HO-1 improves survival of cardiac mouse-to-rat xenografts (16). In human corneal endothelial cells, overexpression of HO-1 promotes angiogenesis and protects cell viability against oxidative stress and toxic effects of chemical irritants (10). A likely mechanism of the ability of HO-1 to protect the corneal cells against oxidant-induced injury is through the generation of the antioxidant bilirubin and the vasodilator CO (9–11, 13).

Another isoform of heme oxygenase is a constitutive enzyme (HO-2; $M_r \cong 34\,000$), which produces biliverdin and CO under normal physiological conditions (12, 13, 17). Please see the review by Maines (4) for more detailed information on these enzymes and factors that affect their expression.

Bilirubin Is a Physiologic Antioxidant

In several studies it was found that different circulating forms of bilirubin are powerful antioxidants: Free bilirubin, albumin-bound bilirubin, conjugated bilirubin, and unconjugated bilirubin were all noted to be effective scavengers of peroxyl radicals and to be able to protect human LDL against peroxidation (6, 13, 18–20).

On the basis of the known involvement of oxidized LDL in the formation of atherogenic plaques and the ability bilirubin to serve as a potent lipid chain-breaking antioxidant under physiological conditions, it was suggested that increased physiological concentrations of plasma bilirubin may reduce atherogenic risk (21, 22). Both animal and human studies have substantiated the suggestion that bilirubin is a physiological antioxidant. Yamaguchi and co-workers (23, 24) isolated and identified oxidative metabolites of bilirubin (biotripyrrins) from the urine of healthy humans and ascorbic acid-deprived rats treated with endotoxin. Feeding of ascorbic acid, a documented physiological antioxidant, reduced the secretion of bilirubin metabolites and suppressed the endotoxin-stimulated hepatic concentration of HO mRNA (24). In another animal model of oxygen radical formation, ischemia-reperfusion of rat liver was noted to induce HO-1 and to promote production of biotripyrrins (6). Again, ascorbic acid feeding attenuated both HO induction and biotripyrrin production in this experimental model (6). These findings imply that bilirubin serves as a physiological antioxidant in ischemia-reperfusion in vivo and that it shares with ascorbic acid a protective function against oxidative stress (6).

Induction of HO was similarly observed in pig hearts, where experimental cardiac ischemia followed by reperfusion was associated with enhanced expression of HO-1 mRNA and increased immunoreactive vascular HO-1 (25). That bilirubin fulfills an antioxidant function in vivo has likewise been shown by Dennery at al. (26) in experiments using Gunn rats exposed to hyperoxia. Further support for the notion that circulating bilirubin functions as an antioxidant that protects against hyperoxidative damage has come from studies of vascular balloon injury that causes oxidative stress and stimulates intimal cell proliferation in rat carotid artery (27). Bilirubin, as well as induction of HO-1 by hemin, prevented this stress-invoked response, suggesting that HO activity and/or bilirubin serve a protective function against injury-mediated proliferation of intimal cells (27).

Human studies have led to similar conclusions that bilirubin production is involved in antioxidant defense mechanisms and that higher bilirubin concentrations are associated with a lower incidence of oxygen radical-mediated injury (28–30). For example, oxidative stress was found to cause depletion of endogenous antioxidants, including bilirubin, in human plasma and to increase production of lipid hydroperoxides (28). Infants with disorders that involve oxygen radical-mediated injury, such as necrotizing enterocolitis, bronchopulmonary dysplasia, intraventricular hemorrhage, and retinopathy of prematurity, display lower circulating bilirubin than healthy controls (29). Likewise, a direct correlation was found between serum bilirubin concentrations and total antioxidant status in premature neonates (30).

Low Plasma Bilirubin as a Risk Factor for CAD

Several studies have noted an inverse relationship between the presence of CAD and circulatory total bilirubin (31, 32). In 1994, Schwertner et al. (31) were the first to observe a significant inverse correlation between total bilirubin plasma concentrations and the prevalence of CAD. This important finding indicated that a lower than normal serum bilirubin concentration is associated with the presence of ischemic heart disease (31). Subsequently, Hopkins et al. (32) noted that patients with early familial CAD have an average total serum bilirubin of 8.9 ± 6.1 $\mu mol/L$ compared with 12.4 ± 8.1 $\mu mol/L$ in healthy control subjects. In a prospective study in middle-aged British men, Breimer et al. (33) observed a U-shaped relationship between circulating bilirubin concentrations and cardiovascular risk, leading to the conclusion that low concentrations of serum bilirubin are associated with increased risk of ischemic heart disease. These and other investigators found that plasma bilirubin correlated inversely with several known risk factors for CAD, such as smoking, LDL-cholesterol, diabetes, and obesity, and correlated directly with the protective factor HDL-cholesterol (34, 35). The reduced concentration of total bilirubin in plasma related univariately and multivariately to the presence of CAD, and this relationship remained significant after adjustment for known CAD risk factors such as age, cholesterol, HDL-cholesterol, smoking, and systolic blood pressure (34). On the basis of these findings, low bilirubin was suggested as an independent risk factor for

CAD, and an inverse correlation was demonstrated between bilirubin concentration and CAD morbidity. Further support for the existence of this inverse correlation came from the work of Hunt et al. *(36)*, who described a genetic variation in bilirubin concentration, with individuals with early CAD displaying lower bilirubin than unaffected persons.

It would be interesting to determine which of the different entities of circulating bilirubin possesses cardioprotective capacity and is associated with the reduced risk for CAD. Antioxidant activity and cardioprotective potential may be attributable to any of the bilirubin forms, including free unconjugated bilirubin, protein-bound unconjugated bilirubin, delta bilirubin, or mono-/diconjugated bilirubin *(19, 22, 37)*. Under physiological conditions, the predominant circulatory form of bilirubin is the unconjugated, albumin-bound form. It is not known whether conditions that modify the relative proportions of this form of bilirubin in the blood, i.e., protein binding, acidosis, hypoxia, and extent of hemolysis, affect the cardioprotective potential of bilirubin. Protein binding, which is modulated by changes in plasma albumin concentrations, the concentrations of drugs that compete on binding, acidosis, and other factors, is expected to affect the balance between free (diffusible) and bound unconjugated bilirubin and thereby change the penetration of unconjugated bilirubin into cells. Likewise, changes in membrane integrity that are induced by hypoxia could potentially modulate the bilirubin transfer capacity of the membrane. In view of these complex interactions, it is important to establish the antioxidative capacity of the different bilirubin forms and to assess how the circulating concentration of free bilirubin, circulating albumin, blood pH, and the presence of drugs modulate bilirubin antioxidative capacity.

Plausible Mechanisms of Bilirubin Action in Prevention of Atherosclerosis

Several mechanisms have been suggested to play a potential role in the antiatherogenic and cardioprotective effects of bilirubin.

BILIRUBIN-MEDIATED INHIBITION OF LIPID OXIDATION

Lipoproteins, and particularly LDL, are highly susceptible to oxidation, and it is known that the atherogenic process involves uptake of oxidized LDL by intimal macrophages, leading to the accumulation of lipid-rich foam cells. Given the antioxidant capacity of bilirubin, it is plausible that bilirubin protects lipids and lipoproteins against oxidation and thereby offers protection against atherogenesis. Accordingly, low bilirubin concentrations may be associated with increases in oxidized lipids and lipoproteins and, therefore, with enhanced atherogenic plaque formation *(31, 38)*.

BILIRUBIN AS REFLECTION OF ENHANCED HO ACTIVITY

Increased HO activity may account for the antiatherogenic and cardioprotective effects of bilirubin through increased elimination of heme and/or enhanced production of CO, iron, and biliverdin. Changes in the concentration of any of these metabolites could affect the pathophysiology of atherosclerosis *(13, 39)*. For example, HO-1-mediated consumption of heme may reduce heme-induced toxic cell injury, and decreased hemoglobin concentrations may enhance vasodilatation. Furthermore, hemoglobin is a scavenger of NO that blunts NO-dependent vasodilatation. CO could affect cardiovascular function through activation of soluble guanylate cyclase and the consequent increase in intracellular cGMP concentrations *(7)*. CO is also an active vasodilator involved in the regulation of vasomotor tone, platelet aggregation, and vascular smooth muscle cell proliferation *(7, 13, 40)*.

Another potential mechanism explaining the association between HO activity and CAD risk may be related to the ability of HO-1 to release iron and change the concentrations of iron stores *(31, 41)*. Mice lacking functional HO-1 develop an anemia associated with abnormally low serum iron concentrations and accumulate iron in the liver and kidney. These iron stores may contribute to tissue injury and chronic inflammation associated with the oxidative damage that characterizes HO-1-deficient animals *(15, 42)*.

The induction of HO by heme is associated with increased expression of ferritin *(43)*. On the basis of this finding it was suggested that iron released through HO activity drives the synthesis of ferritin and that ferritin, by virtue of its iron-binding capacity, provides protection to endothelial cells against oxidative damages *(43)*.

IMMUNE REACTIONS AND INFLAMMATORY PROCESSES

An involvement of bilirubin in immune reactions and inflammatory processes has also been documented. Nakagami et al. *(44)* noted that biliverdin and bilirubin inhibit complement-dependent reactions in vitro and that biliverdin administration inhibits Forssman anaphylaxis in guinea pigs. On the basis of these findings it is possible that bile pigments are endogenous tissue protectors by virtue of their anticomplement activity *(44)*. A correlation between bilirubin metabolism and inflammatory processes is also supported by observations that high HO activity is linked to a faster resolution of inflammation, whereas inhibition of this enzyme appears to potentiate the inflammatory responses *(45)*.

OTHER MECHANISMS

Another possibility is that low bilirubin concentrations per se are not a major causative factor in the development of CAD, but rather a reflection of the presence of this ailment. According to this view, low bilirubin is a result of increased oxidative activity in CAD-prone individuals, leading to consumption of natural antioxidants *(31)*.

Summary

The studies surveyed in this review indicate that free and albumin-bound bilirubin have the potential to serve as lipid antioxidants and oxygen radical scavengers under physiological conditions. Because lipid oxidation and formation of oxygen radicals are involved in the pathophysiology of CAD, a potential antiatherogenic action of circulating bilirubin is conceivable. The evidence presented here indeed confirms that serum bilirubin concentrations in the upper portion of the reference interval for the general population provide protection against CAD, whereas concentrations in the lower portion of the reference interval indicate increased atherogenic risk.

The distinct inverse correlation between plasma bilirubin concentration and CAD morbidity may have important clinical and diagnostic implications. The clinical relevance relates to potential preventive and therapeutic approaches, whereas the diagnostic relevance concerns the diagnostic utility of circulating bilirubin concentrations as a provisional new marker of atherogenic risk that can be measured easily in the clinical laboratory and applied in medical practice. Further studies are undoubtedly warranted to establish the sensitivity and specificity of circulating unconjugated bilirubin as a marker of cardiovascular morbidity.

Although the mechanism(s) underlying the ability of certain bilirubin concentrations to protect against CAD remain to be clarified, it is possible that either the bilirubin concentration itself or changes in the concentrations of other component(s) in the bilirubin synthetic pathway are involved in the protective action. These additional components may include heme, biliverdin, CO, and iron, which are regulated by the activity of HO and have all been implicated in the physiology and pathology of the cardiovascular system. HO is expressed at low basal concentrations in vascular endothelial and smooth muscle cells and is induced by oxidative stress, inflammatory mediators, and oxidized LDL. The complex interactions between HO expression, the circulating concentrations of its substrate and products, and the effect of these components, and specifically of bilirubin, on the vasculature, on lipid metabolism, and on the cardiovascular system will hopefully be the focus of extensive research in the coming years.

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