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REVIEW ARTICLE

Effects of serum bilirubin on atherosclerotic processes

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This review highlights the protective roles of bilirubin against the atherosclerotic process. Bilirubin belongs to the superfamily of tetrapyrrolic compounds formed during heme catabolism. Although for decades bilirubin was considered to be a harmful waste product, recent epidemiologic studies have shown that serum bilirubin levels have consistently been inversely associated with cardiovascular disease (CVD), as well as cardiovascular risk factors such as metabolic syndrome and diabetes. These clinical studies are supported by *in vitro* and *in vivo* experimental data and have demonstrated that bilirubin not only has an ability to scavenge overproduced reactive oxygen species and inhibit vascular smooth muscle cell proliferation but, additionally, has anti-inflammatory effects. In this review, we will discuss the inverse association of serum bilirubin and CVD and cardiovascular risk factors established in various clinical studies. We also review detailed experimental studies about the effect of bilirubin on atherosclerotic processes. *In vitro*, animal and human studies have proved that bilirubin inhibits oxidation of cholesterol which is an important step of atherosclerosis. Bilirubin attenuates chemotactic activity of monocytes and strongly inhibits adhesion of leukocytes to venule and production of pro-inflammatory cytokines. Bilirubin has inhibited serum-driven smooth muscle cell cycle progression at the G1 phase. Lastly, we will discuss briefly the influence of bilirubin on lipoprotein composition and endothelial dysfunction.

Key words: Atherosclerosis, bilirubin, cardiovascular disease

Key messages

- *In vivo* and *in vitro* experimental studies have shown that bilirubin can modulate inflammation, have antioxidant effects, and inhibit smooth muscle cell proliferation.
- Many epidemiologic data also consistently emphasize that bilirubin may play a protective role against atherosclerosis and cardiovascular diseases. In this review, we examine current evidences that bilirubin may have a protective association with atherosclerosis and CVD, mainly demonstrated by clinical studies. Then we also focus on recent progresses in our understanding of the protective mechanism of bilirubin in atherosclerotic disease.

Introduction

Bilirubin is composed of four pyrrole rings connected by carbon bridges formed during heme catabolism. For many years, the bile pigment bilirubin was considered to be a toxic waste product, in particular for central nervous systems. However, more recent evidence suggests that bilirubin may have numerous other beneficial effects, including antioxidant effects, anti-inflammatory action, and direct effects upon cell signalling (1). Many epidemiologic data also consistently emphasize that bilirubin may play a protective role against atherosclerosis and cardiovascular disease (CVD).

In this review, we examine current evidence that bilirubin may have a protective association with atherosclerosis and CVD, mainly demonstrated by clinical studies. We also focus on recent progress in our understanding of the protective mechanism of bilirubin in atherosclerotic disease supported by *in vitro* and *in vivo* experimental studies.

Heme–bilirubin–carbon monoxide metabolic pathway

Bilirubin is the end product of heme catabolism in the systemic circulation. It is formed by the action of heme oxygenase (HO), which is an enzyme that splits cyclic tetrapyrrole heme into biliverdin, carbon monoxide, and ferrous iron. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase.

The organs mainly involved in heme synthesis are the liver and the bone marrow, although every cell including the endothelial cell requires heme to function properly and synthesizes it in the mitochondria. About 75% to 80% of unconjugated bilirubin is derived from hemoglobin released during destruction of senescent red blood cells in the reticuloendothelial

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system (2). Macrophages in connective tissue near vessels also serve as heme sources to the vasculature. Heme in the systemic vasculature can originate from leakage of hemoglobin from red blood cells by oxidative stresses (3). It can also be derived from non-hemoglobin heme proteins from various cells such as hepatocytes and macrophages (Figure 1) (4). The contribution of other hemoproteins (e.g. myoglobin) is of minor importance, due to their slow turnover (2).

Bilirubin, as a non-polar molecule, is solubilized in the vascular bed by binding to albumin. When reaching the liver, it is actively transported by the basolateral ATP transporter, solubilized in the cytoplasm by binding to specific binding proteins, and subsequently conjugated by the action of bilirubin UDP-glucuronosyltransferase (UGT) with two molecules of glucuronic acid. Bisglucuronosyl bilirubin is then actively secreted into the bile and proceeds into the intestinal lumen, where it undergoes further metabolic changes. From the metabolic point of view, there are several crucial enzymatic steps which play an important role in bilirubin homeostasis with subsequent impacts on the risk of metabolic diseases, including cardiovascular disease, diabetes, metabolic syndrome, hypertension, and obesity.

The serum bilirubin level is regulated by many factors. As noted above, HO and UGT are important steps in bilirubin production. Actually in Gilbert syndrome, in which UGT is partially deficient, mild unconjugated hyperbilirubinemia in the range of 1.2–6.0 mg/dL is a typical presentation (5). HO-1 is induced by stimuli provoking oxidative stress, including free oxygen radicals and bacterial lipopolysaccharides (6,7). HO-1 is also induced by increases in the hepatic heme pool induced by various drugs, natural compounds, inflammatory cytokines, and growth factors (8,9).

Biological properties of bilirubin

Bilirubin was considered to be a waste product of the HO action. Little attention was paid to the physiological roles of these products, until the study in 1987 showing that bilirubin suppresses the oxidation of peroxyl radicals more than does α-tocopherol (10). After this, numerous subsequent studies confirmed that bilirubin is one of nature's most potent antioxidants. Indeed, unconjugated bilirubin (UCB) concentrations as low as 10 nmol/L have been reported to protect neuronal cultures from the oxidative stress generated by 10,000 times higher concentrations of hydrogen peroxide (11). Notably, the biliverdin–bilirubin redox cycle, in which bilirubin is continuously regenerated by biliverdin reductase (BVR) and reutilized, enables such low concentrations of UCB to protect against vastly higher concentration of hydrogen peroxide (11). These antioxidant activities from *in vitro* experimental findings have been reproduced in clinical observations comparing subjects with Gilbert syndrome with a control. Plasma UCB levels are directly correlated with serum and plasma total antioxidant capacity (12). Furthermore, serum bilirubin has been demonstrated to correlate negatively with urinary markers of oxidative stress (13).

In addition, bilirubin has been proven to have anti-inflammatory properties (1). UCB prevented tumour necrosis factor (TNF)-α-induced overexpression of adhesion molecules such as E-selectin vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells *in vitro* (14). There is also a human study on subjects with Gilbert syndrome demonstrating that serum bilirubin levels are associated inversely with soluble forms of CD40 ligand and P-selectin (15). Besides inhibiting expression of inflammatory proteins, bilirubin has been shown to have

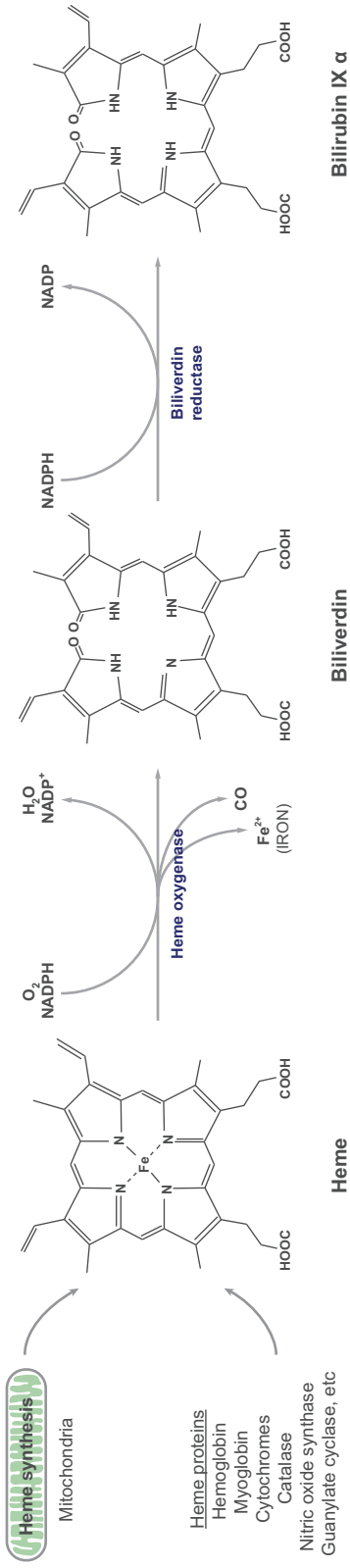

Figure 1. The enzyme heme oxygenase catalyses the breakdown of heme to the products biliverdin, carbon dioxide, and iron. Biliverdin reductase converts biliverdin to bilirubin. NADP = nicotinamide adenine dinucleotide phosphate; NADPH = reduced form of NADP; CO = carbon dioxide; Fe^{2+} = iron(II).

anti-complement properties. UCB interferes with the interaction between C1q and immunoglobulins, thus inhibiting the initial step in the activation of complement through the classical pathway (16). In accordance with these data, many studies have shown a negative relationship between bilirubin and inflammatory markers, such as high-sensitivity C-reactive protein (hs-CRP) levels (1,17,18).

Bilirubin: protection against cardiovascular diseases (CVD)

Bilirubin and coronary artery disease (CAD)

The first report that serum bilirubin might be inversely related to coronary artery disease (CAD) was issued in 1994 (19). In that study, a 50% decrease in total bilirubin was associated with a 47% increase in the odds of having more severe CAD, proven by coronary angiography. After that study, Hopkins et al. found that bilirubin was an independent protective factor with an odds ratio (OR) of 0.25 for an increase of 1 mg/dL when comparing patients with early familial CAD with control subjects (20). These relationships were proved in asymptomatic males who had abnormal treadmill tests and who underwent coronary angiography to rule out the presence of CAD. The reduced level of total bilirubin was correlated univariately and multivariately with the presence of CAD, and this relationship remained significant after adjustment for known CAD risk factors such as age, cholesterol, high-density lipoprotein (HDL) cholesterol, smoking, and systolic blood pressure (21). On the basis of these findings, one study examined the predictive ability of bilirubin for coronary artery disease which is confirmed by angiography. Bilirubin-containing ratios were found to be an independent risk predictor when tested with the traditional risk factors (22).

In addition to stenosis proved by coronary angiography, bilirubin has also been proved to be associated with coronary artery calcification (CAC) which is a good surrogate marker of the presence and amount of coronary atherosclerosis. In a recent cross-sectional study of 398 men and 239 women, serum bilirubin concentrations were found to be strongly related to CAC scores and independent determinants of CAC in both men and women (23). Although CAC is a good surrogate marker for coronary atherosclerosis, non-calcified coronary plaque, which is more vulnerable to rupture and atherosclerotic events, cannot be detected by CAC. In this case, CT coronary angiography can be used to detect coronary plaque. A recent cross-sectional study conducted on 2682 men who were free of CAD and who underwent coronary CT revealed that bilirubin was inversely associated with total coronary plaque (24).

Besides anatomic evidence of atherosclerosis, bilirubin has been investigated for association with functional markers such as coronary flow reserve (CFR) measured by echocardiography. CFR is used to assess epicardial coronary arteries and to examine the integrity of coronary microvascular circulation. Gullu et al. investigated 160 young (18–45 years) healthy subjects without CAD using echocardiography. CFR values measured by stress echocardiography were significantly higher in subjects with high bilirubin concentrations than in those who were in the low bilirubin groups (25). This study has confirmed that serum bilirubin concentration in the upper portion of the reference interval provides protection against coronary microvascular dysfunction and CFR impairment.

The inverse association between serum bilirubin concentrations and CAD has been found in several prospective studies (26). In the Framingham Offspring Study involving 4276 men and women, higher serum bilirubin concentrations were found to be associated with a lower risk of myocardial infarction, coronary death, and any cardiovascular events in men; however, the inverse association was only suggestive in women (27). Another recent study investigated CAD prevalence in patients with Gilbert syndrome. Gilbert syndrome is caused by partial deficiency of bilirubin UGT in the liver tissue and is characterized by sustained unconjugated hyperbilirubinemia (5). In this study the prevalence of CAD in patients with Gilbert syndrome was 2% (0.05%–10.7%, 95% CI), compared to 12.1% in the general population ($P < 0.05$) (12).

In addition, a meta-analysis study of 11 studies has shown that serum bilirubin concentrations are inversely associated with the severity of atherosclerosis in men ($r = -0.31$, $P < 0.0001$). Non-parametric, regression, and stratified analyses all showed an inverse and dose-dependent relationship between serum bilirubin concentrations and different types and severities of cardiovascular disease (CVD). In this study each 1.0 μmol/L increase in serum bilirubin was found to be associated with a 6.5% decrease in CVD (28).

Bilirubin, stroke, and other forms of cardiovascular disease (CVD)

A similar inverse association has been shown between serum bilirubin concentrations and stroke and carotid intimal-medial thickening. Kimm et al. found that men with higher serum bilirubin levels had a lower hazard ratio for ischaemic stroke after adjustment for multiple confounding risk factors compared with men in the lowest bilirubin quartile (OR 0.66; 95% confidence interval (CI) 0.49–0.89, $P = 0.0016$) (29). Perlstein et al. had similar findings in a cross-sectional cohort study that consisted of 7075 adults enrolled in the National Health and Nutrition Examination Survey (NHANES) and included both sexes and various racial groups. In their study, they found that each 0.1 mg/dL increase in serum bilirubin led to a 9% reduced odds of stroke (OR 0.90; 95% CI 0.86–0.96) (30).

An inverse relationship between serum bilirubin and the risk of atherosclerosis of the carotid artery and the peripheral artery was also found in numerous studies. Vitek et al. found that the mean intima-media thickness (IMT) in hyperbilirubinemic subjects as compared with controls was substantially lower ($P = 0.017$), and hyperbilirubinemic men also had very low age-adjusted prevalence odds ratios for having an IMT above the 50th percentile of controls, even after adjustment for selected vascular risk factors ($P = 0.034$) (31). Erdogan et al. also found that carotid IMT was significantly greater in subjects with lower serum bilirubin concentrations (0.5 ± 0.13 mm versus 0.42 ± 0.07 mm, $P < 0.0001$) (32). The relationship between bilirubin and carotid IMT, a marker of subclinical atherosclerosis, in non-diabetic and diabetic subjects was evaluated by Dullaart et al. In this study, carotid IMT was negatively related to bilirubin after adjustment for cardiovascular risk factors in both non-diabetic and in diabetic subjects (33).

Perlstein et al. reported inverse association of bilirubin with peripheral artery disease (PAD) in the NHANES (1999 to 2004). In that study a 0.1 mg/dL increase in bilirubin level was associated with a 6% reduction in the odds of PAD (OR 0.94; 95% CI 0.90–0.98) after adjustment for cardiovascular risk factors (34). The inverse association of bilirubin with PAD tended to be stronger among men (OR 0.90; 95% CI 0.85–0.96) compared with women (OR 0.97; 95% CI 0.91–1.04).

Bilirubin, diabetes, and metabolic syndrome

There are also numerous studies indicating serum bilirubin levels being associated with metabolic syndrome (MS) or diabetes. The

first report was about the association of bilirubin with metabolic syndrome among children and adolescents. The prevalence of MS was from 6.6 ± 1.2% in the lowest quartile to 2.1 ± 1.9% in the highest quartile of concentration of total bilirubin. This graded association remained significant after the adjustment of other covariates (35). From NHANES between 1999 and 2006, 15,876 participants were selected for study. After age adjustment, increased total bilirubin was associated with a 26% reduction in diabetes risk (OR 0.74; 95% CI 0.64–0.88). Multivariate analysis, adjusting for all diabetes risk factors assessed, confirmed this association (OR 0.80; 95% CI 0.67–0.95) (36). In a prospective cohort study for 5 years in patients with MS, bilirubin was associated with an adverse outcome ($P < 0.001$). In a multivariate Cox regression analysis, serum bilirubin, in addition to age and basal superoxide generation of circulating mononuclear cells, was also an independent predictor of total adverse events (HR 0.002; 95% CI 0.000–0.520) (37). Another study analysing NHANES 1999–2008 revealed that subjects taking statin ($n = 1156$) had lower total bilirubin levels than those not taking any lipid-lowering medication ($n = 2134$) after adjusting for age, sex, race/ethnicity, and survey period (adjusted mean = 0.699 versus 0.729 mg/dL, respectively, $P = 0.001$) (38).

Bilirubin: influence on atherosclerotic processes

Atherosclerosis is an inflammatory disease in which lipid deposition in the arterial wall, resulting from elevated levels of plasma cholesterol, is central to lesion development. This process involves the uptake of modified low-density lipoprotein (LDL) cholesterol by macrophages and is associated with a state of heightened oxidative stress and damage (38,39). As atherosclerotic lesions progress, migration and proliferation of smooth muscle cells and deposition of fibrous tissue lead to an advanced, complicated lesion (40).

Numerous evidences have demonstrated that bilirubin can affect various steps in the progression of atherosclerosis and can attenuate the atherosclerotic process (Table I). In this section we discuss our present knowledge about the possible protective role of bilirubin in the progression of atherosclerosis.

Bilirubin and the atherosclerotic process—antioxidant protection

As previously reviewed, bilirubin is an efficient *in vitro* scavenger of different types of oxidants, and there is increasing evidence that this translates into cellular activity (10,11,40,41). For example, when added at micromolar concentrations to cell culture media, bilirubin protects various cells, including endothelial and smooth muscle cells, against toxicity induced by hydrogen peroxide (42). In cells in which HO-1 is induced and exogenous hemin is provided as a substrate, increased resistance to oxidant-mediated toxicity is observed only while bilirubin formation takes place (42). Bilirubin has also been shown to have powerful antioxidant activity against oxidation of lipoproteins, which is one of the important steps of plaque formation and atherosclerosis. Indeed, bilirubin has been demonstrated to be almost 30 times more potent towards the prevention of LDL oxidation compared to a vitamin E analogue, Trolox, which represents a lipid-soluble antioxidant substance (43). It has also been noted that bilirubin is more effective at protecting lipids from oxidation than the water-soluble antioxidants such as glutathione, which primarily protects proteins from oxidation (44). Evidence for an *in vivo* antioxidant function of bilirubin has also been published. Using Gunn rats as an experimental model of hyperbilirubinemia, Dennery et al. have shown that the plasma of jaundiced rats exposed to hyperoxia shows fewer signs of oxidative damage than plasma from corresponding control animals (45).

In a human study, Benitez et al. found that increases in the concentration of uric acid, bilirubin, and ascorbic acid after aerobic exercise in 11 male athletes result in a significant increase in total antioxidant serum capacity measured by reverse-phase chromatography, which can measure the oxidation of LDL and HDL cholesterol (46).

Bilirubin and the atherosclerotic process—inflammation

There is ample evidence that HO-1, which catalyses the rate-limiting step of heme catabolism, is important in regulating inflammation *in vivo* (40). Ishikawa et al. have reported that inducible HO-1 is highly induced by oxidized LDL. This augmented HO-1 induction resulted in the reduction of monocyte chemotaxis in response to LDL oxidation. Furthermore, pretreatment with bilirubin, the product of HO, further reduced chemotaxis of monocytes (47). Similarly, chemotactic activity stimulated by angiotensin II was suppressed by HO activity and accumulation of bilirubin in monocytes. Notably, exogenously applied bilirubin and carbon monoxide mimicked the inhibitory effect of HO-1 on the chemotactic response (48). Interestingly, the migration of isolated murine splenic lymphocytes across monolayers of murine endothelial cell lines is significantly inhibited by the physiological concentration of bilirubin, in the absence of an effect on adhesion (49). These results suggest that bilirubin blocks VCAM-1-dependent lymphocyte migration *in vitro* and support a potential role for bilirubin as an endogenous immunomodulatory agent (49).

In addition, it has been suggested that bilirubin and HO-1 simultaneously affect the activity of macrophages. Expression of HO-1 induction and bilirubin IXα production in foam cells in atherosclerotic regions of hypercholesterolemic rabbits was proved by *in situ* hybridization and immunohistochemistry. These results provide the first *in vivo* evidence of the co-localization of HO-1 and bilirubin IXα in foam cells, suggesting a role of HO-1 induction and bilirubin in the modulation of macrophage activation in atherosclerosis (50).

An *in vivo* study using a rat model showed that induction of the HO-1 activity and generation of bilirubin serve as a strong inhibitor of leukocyte adhesion in venule. These results suggest that HO-1 and bilirubin serve as a potential stratagem to prevent oxidant-induced microvascular leukocyte adhesion (51). Bilirubin from HO-1 also attenuated vascular endothelial activation and dysfunction. HO-1 induction and bilirubin production by vascular endothelial cells significantly attenuated the production of VCAM-1, monocyte chemotactic protein-1, and macrophage colony-stimulating factor. In addition, HO-1 overexpression alleviated endothelial dysfunction as judged by restoration of attenuated endothelial nitric oxide (NO) synthase expression after exposure to oxidized LDL and TNF-α (52). These effects were also observed by treatment with bilirubin, not by carbon monoxide. These results suggest that the antiatherogenic properties of HO-1 may be mediated predominantly through the action of bilirubin by inhibition of vascular endothelial activation and dysfunction in response to proinflammatory stresses.

There is also much evidence that bilirubin affects cell signal transductions. Bilirubin has prevented the nuclear translocation of nuclear factor (NF)-kappaB induced by TNF-α. By inhibition of the NF-kappaB transduction pathway, bilirubin blunted the overexpression of adhesion molecules in endothelial cells. Collectively these data may contribute to explain the protective effect of bilirubin against development of atherosclerosis (53). Bilirubin blunted the TNF-α-induced gene upregulation of E-selectin, VCAM-1 and ICAM-1 in endothelial cell lines *in vitro*. It also inhibited the polymorphonuclear

Table I. Evidences for the protective role of bilirubin against atherosclerosis.

Related process	Type	Outcomes	Ref.
Study characteristics			
Antioxidant	*In vitro*	Bilirubin, at micromolar concentrations, efficiently scavenges peroxyl radicals generated chemically. In liposomes, bilirubin suppresses the oxidation more than does α-tocopherol	10
	In vitro	Depletion of bilirubin by RNA interference markedly augments tissue levels of reactive oxygen species and causes apoptotic cell decrease. Antioxidant capacity was greater than that of glutathione	11
	In vitro	UCB, at the normal serum level, inhibits oxidation of LDL cholesterol. UCB is more than 20 times more effective than trolox in preventing LDL oxidation.	43
	In vivo, animal	Mice with depletion of HO-2 display greater lipid than protein oxidation. RNA interference depletion of BVR increases oxidation of lipids more than protein.	44
	In vivo, animal	Serum bilirubin protects against serum oxidative damage in the first days of life in neonatal Gunn rats exposed to hyperoxia. Serum lipid hydroperoxides in jaundiced rats was lower than that in non-jaundiced rats	45
	In vivo, human	Increases in the concentration of uric acid, bilirubin, and ascorbic acids after aerobic exercise result in a significant increase in total antioxidant serum capacity	46
Inflammation	*In vitro*	HO-1 induction or pretreatment with bilirubin resulted in the reduction of monocyte chemotaxis in response to LDL oxidation	47
	In vitro	Enhanced HO activity with the increase of bilirubin suppresses not only Ag II-stimulated superoxide formation, but also Ag II-enhanced chemotactic activity	48
	In vivo, animal	Bilirubin blocks VCAM-1-dependent lymphocyte migration *in vitro* and ameliorates VCAM-1-mediated airway inflammation *in vivo* in a murine asthma model	49
	In vivo, animal	Induction of HO-1 in endothelial cells and foam cells and accumulation of bilirubin in foam cells were observed in atherosclerotic lesions of hypercholesterolemic rabbits	50
	In vivo, animal	Induction of HO-1 or superfusion with bilirubin at the micromolar level decreases rolling and adherent responses of leukocytes in venule which is provoked by oxidative stress and ischaemia-reperfusion in rats	51
	In vivo, animal	Induction of HO-1 or bilirubin treatment attenuated proinflammatory responses and improved impaired endothelium-dependent vascular relaxation response in thoracic aorta from high-fat-fed LDL receptor knock-out mice	52
	In vitro	UCB attenuates overexpression of adhesion molecules via inhibition of the NF-kB transduction pathway	53
	In vitro	UCB limits the overexpression of adhesion molecules and inhibits the PMN endothelial adhesion induced by the TNF-α in endothelial cell lines	14
Smooth muscle cell proliferation	*In vivo*, animal	Bilirubin inhibited VSMC cycle progression at the G1 phase and prevented intimal hyperplasia after balloon injury in hyperbilirubinemic Gunn rats	54
	In vivo, animal	Bilirubin reduced phosphorylation of p38 MAPK and JNK1/2 in VSMC and inhibited vascular stenosis after balloon injury in hyperbilirubinemic Gunn rats	55
	In vivo, animal	Local administration of bilirubin immediately following balloon injury of rat carotid arteries attenuated neointimal formation. Bilirubin significantly decreases in ERK activity in injured blood vessels	56
	In vitro	Bilirubin inhibits growth of proliferating human coronary artery smooth muscle cells and causes the transition from proliferative to contractile phenotype	57
	In vivo, human	Total bilirubin levels were negatively associated with ISR in 1076 patients who underwent coronary stenting and follow-up angiography	58
Endothelial dysfunction	*In vivo*, animal	Induction of HO-1 or administration of biliverdin can prevent endothelial cell sloughing in diabetic rats	59
	In vitro	Administration bilirubin attenuated mitochondrial dysfunction, caspase-3 activation, and cell death caused by the knockdown of HO-1 in human endothelial cells	60
	In vivo, human	Log-transformed total bilirubin is positively correlated with the change in coronary artery diameter to intra-coronary papaverine administration, which reflects the endothelial function in overweight patients who underwent coronary flow studies	18

Ag = angiotensin; BVR = biliverdin reductase; ERK = extracellular signal-regulated kinases; HO = heme oxygenase; ISR = in-stent restenosis; JNK = c-Jun N-terminal kinases; LDL = low-density lipoprotein; MAPK = mitogen-activated protein kinases; NF-kB = nuclear factor-kappa B; PMN = polymorphonuclear leukocytes; TNF-α = tumour necrosis factor-α; UCB = unconjugated bilirubin; VCAM-1 = vascular cell adhesion molecule 1; VSMC = vascular smooth muscle cells.

leukocyte endothelial adhesion. These data contribute further to explain the protective effect of bilirubin against development of atherosclerosis (14).

Bilirubin and the atherosclerotic process—smooth muscle cell proliferation

In addition to influencing the inflammatory processes, bilirubin can prevent neointimal formation by inhibiting the proliferation of smooth muscle cells. For example, bilirubin has been shown to attenuate balloon injury-induced neointimal formation in Gunn rat and in wild-type rat models (54). *In vitro* bilirubin and biliverdin have inhibited serum-driven smooth muscle cell cycle progression at the G1 phase via inhibition of the mitogen-activated protein kinase signal transduction pathways and inhibition of phosphorylation of the retinoblastoma tumour suppressor protein (54). The same authors also demonstrated that bilirubin and biliverdin caused p53-dependent cell cycle arrest by hypophosphorylation of the retinoblastoma tumour suppressor protein in growth factor-stimulated vascular smooth muscle cells in a balloon injury model in rats (55). Another study has shown that local administration of bilirubin attenuates neointimal formation following injury of rat carotid arteries and regulates the proliferation and migration of human arterial smooth muscle cells (56). In this study bilirubin inhibits neointimal formation after arterial injury, and this is associated with alteration in the expression of cell cycle regulatory proteins. Furthermore, bilirubin blocks proliferation and migration of human arterial smooth muscle cells and arrests smooth muscle cells in the G0/G1 phase of the cell cycle (56).

It is also known that bilirubin affects other cell signalling pathways to inhibit smooth muscle cell proliferation. Stoeckius et al. further investigated the molecular events by which bilirubin inhibits growth of proliferation of human coronary artery smooth muscle cells (57). In this study bilirubin impaired the activation of the Raf/ERK/MAPK pathway and the cellular Raf and cyclin D1 content that results in retinoblastoma protein hypophosphorylation on amino acids S608 and S780. These events impede the release of YY1 to the nuclei and calcium-dependent YY1 proteolysis in human vascular cells. They concluded that in the serum-stimulated human vascular smooth muscle primary cell cultures bilirubin favours growth arrest, and proposed that this activity is regulated by its interaction with the Raf/ERK/MAPK pathway, the effect on cyclin D1 and Raf content, the altered retinoblastoma protein profile of hypophosphorylation, calcium influx, and YY1 proteolysis. These observations provide important mechanistic insight into the molecular mechanism underlying the transition of human vascular smooth muscle cells from proliferative to contractile phenotype and the role of bilirubin in this transition (57).

These inhibitory effects of bilirubin of neointimal formation have also been demonstrated in a human study. The main mechanism of in-stent restenosis (ISR) is known as neointimal formation. Kuwano et al. investigated 1076 consecutive patients who underwent coronary stenting and follow-up angiography. In this study the ISR rate at follow-up is significantly correlated with the total bilirubin level. A significant negative correlation between the total bilirubin and ISR was revealed by multivariate analysis (OR 0.6; 95% CI 0.39–0.89) (58).

Bilirubin and the atherosclerotic process—endothelial cell dysfunction

Bilirubin can also inhibit endothelial dysfunction. It has been reported to prevent endothelial cell sloughing caused by hyperglycaemia. In an animal model of streptozotocin-induced diabetes, administration of bilirubin prevented hyperglycaemia-induced endothelial cell sloughing via a decrease in oxidative stress. These results demonstrate that bilirubin may be a novel approach to prophylactic vascular protection in diabetes (59). Furthermore, bilirubin may prevent endothelial dysfunction due to oxidative stresses. Treatment of endothelial cells with HOCl stimulated mitochondrial dysfunction, caspase-3 activation, and cell death. Administration of bilirubin reversed these actions (60). These results suggest that bilirubin may represent a critical adaptive response to maintain endothelial cell viability at sites of vascular inflammation and atherosclerosis (60).

In a human study Yoshino et al. investigated 107 patients without coronary heart disease who underwent coronary flow studies. Coronary dilatation following papaverine injection during coronary angiography was measured to assess the effects of bilirubin on endothelial function. In this study log-transformed total bilirubin is positively correlated with flow-mediated dilatation, suggesting that a high bilirubin level is associated with favourable coronary endothelial function (18).

Bilirubin and the atherosclerotic process—influence on lipoproteins

Uptake of modified LDL cholesterol by macrophage is an important step towards progression of atherosclerosis. An LDL particle contains a single apolipoprotein B-100 molecule along with 80 to 100 additional ancillary proteins (61). Bilirubin may also affect composition of lipoproteins. One study has revealed that bilirubin is significantly correlated with apolipoprotein B, which is a better predictor than LDL cholesterol level (62). Upon receiver operator characteristic curve (ROC) analysis, bilirubin showed an inverse relationship with risk of CAD, with the area under the ROC curve comparable to lipoprotein (62). O'Kane et al. compared serum lipids and the apolipoprotein levels of patients with primary biliary cirrhosis with those of a normal control. The level of apolipoprotein A1, which protects against atherosclerosis, is higher in the elevated bilirubin group (63).

Serum amyloid A (SAA) proteins, a family of apolipoproteins associated with HDL, are produced by the liver during the acute phase of inflammation. They are implicated in several chronic inflammatory diseases, including atherosclerosis (64). Higher SAA levels impair HDL antioxidative functionality (65). Bilirubin was correlated inversely with SAA levels in subjects without metabolic syndrome (66). These raise the possibility that metabolic syndrome may elicit abnormalities in HDL antioxidative function, which could mask a relationship between SAA and bilirubin. This was supported by the fact that of the individual metabolic syndrome components, the strongest effect, modification of bilirubin on SAA, was observed for HDL cholesterol.

Remnant-like particle (RLP) cholesterol is an independent cardiovascular disease risk factor in women according to the results from the Framingham heart study (67). The relationship between serum bilirubin level and serum RLP cholesterol was investigated in 270 males who visited the outpatient clinic. It was found that the serum RLP cholesterol level was significantly lower in patients in the high bilirubin group than in those in the lower bilirubin group. These findings suggest that serum bilirubin within physiological levels may affect the serum RLP cholesterol and may play a role in preventing atherosclerosis. It is not clear, however, how bilirubin makes these changes of lipoproteins.

Bilirubin and the atherosclerotic process—heme oxygenase

HO, which is the initial, rate-limiting step of heme degradation, is well known to have a protective function of the enzyme in a

variety conditions associated with cellular stress and pathologies, and this has been the subject of excellent reviews (9,40,68).

HO-1 is expressed most strongly in tissues involved in erythrocyte or hemoglobin metabolism, whereas in most other tissues HO-1 typically occurs at low basal levels but responds rapidly by transcriptional activation to diverse stimuli (40). A number of stressors (9), such as increased blood pressure, altered laminar flow in blood vessels, advanced glycation end products, smoking, oxidized lipids, and a multitude of systemic inflammatory processes, lead to increased cellular HO-1 expression.

The cardioprotective role of HO has been developed and substantiated significantly in experimental models of atherosclerotic vascular disease, including atherosclerosis, intimal hyperplasia, and myocardial infarction (40). When compared with HO-$1^{+/+}$apoE$^{-/-}$ mice, HO-$1^{-/-}$apoE$^{-/-}$ mice have shown an accelerated and more advanced atherosclerotic lesion formation (69). Furthermore, vascular smooth muscle cells isolated from HO-$1^{-/-}$ mice were more susceptible to oxidant stress, leading to more cell death. These data demonstrate that HO-1 plays an essential, protective role in the vascular setting of hypercholesterolemia and vascular stenosis. HO-1 directly reduces vasoconstriction and inhibits cell proliferation during vascular injury (70). The effects of HO-1 on vascular smooth muscle cell growth and vascular relaxation are mediated by cell cycle arrest involving $p21^{Clp1}$ and by guanylate cyclase and cGMP, independently of NO, respectively (70). In experimental research using cardiac-specific transgenic mice overexpressing different levels of HO-1, hearts from transgenic mice have shown improved recovery of contractile performance during reperfusion after ischaemia in an HO-1 dose-dependent manner (71). These myocardial ischaemia and reperfusion experiments have shown that infarct size was only 14.7% of the area at risk in transgenic mice compared with 56.5% in wild-type mice (71). These data have demonstrated that overexpression of HO-1 in the cardiomyocyte protects against ischaemia and reperfusion injury, thus improving the recovery of cardiac function.

There is overall support for the concept that HO-1 is important in regulating inflammation *in vivo*. Compared with wild-type mice, HO-$1^{-/-}$ mice develop chronic inflammation with increasing age, characterized by enlarged spleen and lymph nodes, hepatic inflammatory infiltrates, and high peripheral white blood cell counts (72). It has also been noted that HO-$1^{-/-}$ mice show the hallmarks of vascular injury, indicated by monocytes adhering to vessel walls (72). Other interesting studies have shown that protoporphyrin IX induction of HO-1 resulted in decrease in IL-1β, IL-6, and TNF-α concentrations, joint swelling, cartilage degradation, and proliferation of inflammatory tissue in joints of mice with non-immune arthritis (73). These reports suggest that HO-1 contributes to physiological homeostasis and that in the absence of HO-1 the potential for a proinflammatory environment develops.

Carbon monoxide, one of three main by-products of the catabolism of heme by HO, also exerts potent anti-inflammatory effects and mediates much of the anti-inflammatory effects seen with HO-1 (74). Carbon monoxide at low concentrations inhibited the expression of proinflammatory cytokines and increased the anti-inflammatory cytokine interleukin-10 both *in vitro* and *in vivo* (74). Along with biliverdin and bilirubin, carbon monoxide is an important product of HO for anti-inflammatory reaction.

Conclusion and perspectives

In this review, the numerous experimental as well as clinical studies on the association between CVD and bilirubin and on the effects of bilirubin on the atherosclerotic process have been discussed. Protective properties of bilirubin are implicated in each important step of the atherosclerosis progression (Figure 2). Clinical studies about the association between bilirubin and CVD argued that low serum bilirubin concentrations are associated with an increased risk of atherosclerotic diseases. These studies, however, include only cross-sectional studies or retrospective or

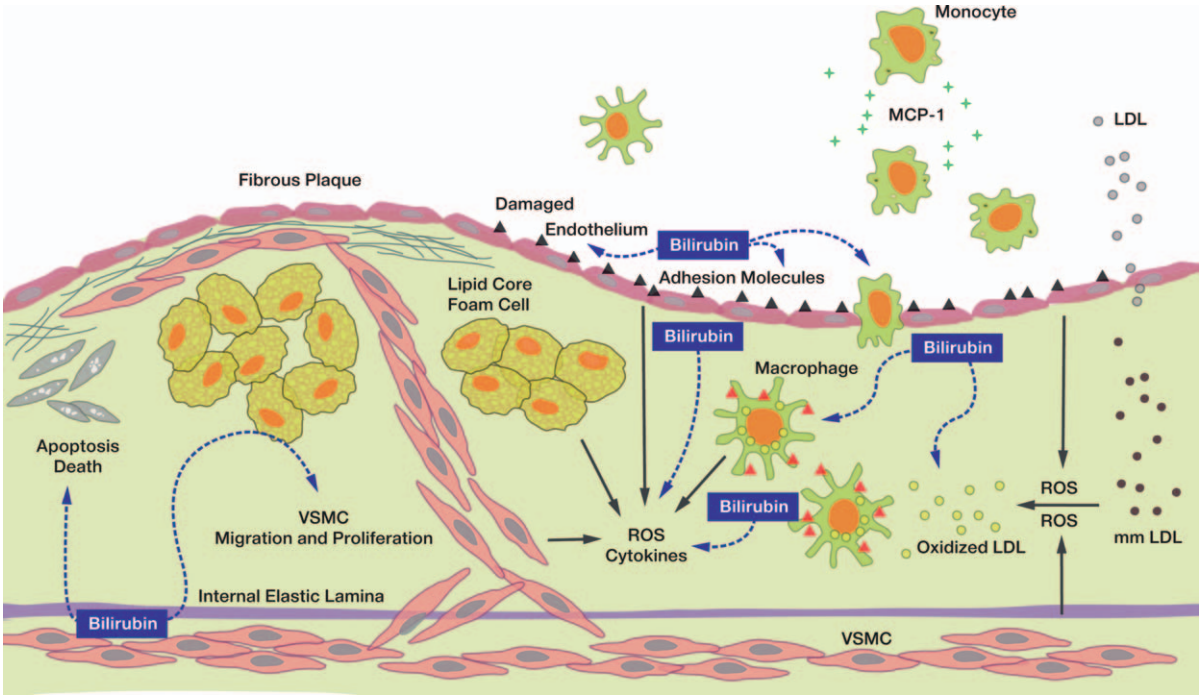

Figure 2. Protective role of bilirubin against atherosclerosis. Oxidation of LDL and ROS itself involves atherosclerosis progression. Bilirubin has antioxidant properties, inhibits monocyte chemotaxis, attenuates expression of adhesion molecules on endothelial cells, improves endothelial dysfunction, and inhibits proliferation of VSMCs. Protective properties of bilirubin in the atherosclerotic processes are indicated as blue dotted arrows. LDL = low-density lipoprotein; MCP-1 = monocyte chemotactic protein-1; ROS = reactive oxygen species; VSMC = vascular smooth muscle cell.

prospective cohort studies (26). We could not conclude a strong causal relationship between bilirubin and CVD based on these studies. To verify a causal relationship, intervention which can increase the bilirubin level in serum or tissue, such as an HO inducer, may be tested for prevention or suppression of the progress of atherosclerosis. Translational studies are also required to find new options influencing bilirubin production or controlling bilirubin levels.

It is uncertain whether low bilirubin levels are associated with CVD mortality. If so, serum bilirubin concentration can be used for risk stratification of individual patients and prevention of CVD death. Stender et al. tested the hypothesis that elevated plasma bilirubin is causally related to decreased risk of ischaemic heart disease and myocardial infarction by using a Mendelian randomization approach and meta-analysis of three independent studies from Copenhagen, Denmark: The Copenhagen General Population Study, The Copenhagen City Heart Study, and The Copenhagen Ischaemic Heart Disease Study. They did not observe an association between genetically elevated bilirubin and risk of ischaemic heart disease or myocardial infarction (75). Further prospective studies are to be performed to determine whether elevated serum bilirubin concentrations are associated with decreased CVD mortality and whether preventive strategies based on stratification according to bilirubin level are effective.

A genetic basis for association between bilirubin and CVD was sought in several studies (76–80). It is well known that individuals with the UGT1A1*28 allele have approximately one-third of the risk of CVD and CAD and have significantly higher serum bilirubin concentration than those with wild-type allele (80). Recently metagenomic studies have become very popular, and one study has demonstrated that the *Bifidobacterium* species in milk and faeces is associated with lower bilirubin levels in patients with breast milk jaundice (81). Furthermore, analysis of the gut microbiota of mice fed a high-fat diet has proved that an increase of the *Clostridium* species increases the conversion of bilirubin to urobilinogen (82). Because gut flora plays an important role in the metabolism of bilirubin, further metagenomic studies may identify which microbiota is significantly associated with bilirubin concentration and the risk of CVD. Further genetic studies should also be performed to find an association between the HO-1, UGT1A1 polymorphism, serum bilirubin concentrations, and CVD mortality.

In vivo experimental studies have shown that bilirubin can modulate inflammation, have antioxidant effects, and inhibit smooth muscle cell proliferation. Very few studies, however, have been performed to determine whether bilirubin or HO-1 induction may alleviate some of the acute complications associated with coronary artery bypass or prevent major CVD in high-risk subjects in animal models. Such studies offer great promise and could have an impact on the health of individuals with low serum bilirubin levels and at risk for CVD as therapeutic tools or preventive measures.

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