Biological Properties and Therapeutic Potential of Bilirubin

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Abstract: Bilirubin was long considered a useless metabolite of heme catabolism, responsible for the clinical manifestation of jaundice, and potentially toxic in high doses, particularly in neonates. In the past two decades the potent biological properties of bilirubin, particularly as an antioxidant, have been recognised, and this has prompted a number of investigations into this molecule concerning its *in vitro* and *in vivo* properties. This review summarises that work, as well as more recent investigations into the potential therapeutic uses of bilirubin.

Keywords: bilirubin, heme, antioxidant.

INTRODUCTION

Bilirubin is a conjugated tetrapyrroledicarboxylic acid, and is the principle pigment in bile. Bilirubin is the end product of heme catabolism in mammals, and most of the circulating bilirubin derives from senescent erythrocytes. At the end of the normal life span of erythrocytes, the heme dissociates from hemoglobin and is oxidised by the membrane-bound enzyme heme oxygenase (EC 1.14.99.3) to biliverdin, producing carbon monoxide as a by-product. Subsequent metabolism of biliverdin by the cytosolic enzyme biliverdin reductase (EC 1.3.1.24) gives rise to bilirubin (Fig. (**1**)). Most of the bilirubin produced in the body is mono- or di-glucuronidated in the liver by a glucuronyl transferase enzyme, and these water-soluble products are excreted in the bile. Bilirubin is obtained industrially by extraction of either cattle or pig bile, and it can be isolated as light-sensitive orange-red crystals. In humans, accumulation of bilirubin in the bloodstream causes yellow pigmentation of the plasma, in turn causing the skin and sclerae to become yellow, appearing clinically as jaundice. Normal human serum levels of bilirubin are in the range 5-17 μM, with levels above around 43 μM manifesting as jaundice [1].

Bilirubin exists in the serum in four major forms: as unconjugated bilirubin, as the monoglucuronide, as the diglucuronide, and as albumin-bound bilirubin. Weiss *et al.* showed that albumin-bound bilirubin constituted from 8 to 90% of total bilirubin in patients with jaundice or with Dubin-Johnson syndrome, but could not be detected in healthy volunteers, indicating a build-up in serum of conjugated bilirubin when hepatic excretion was impaired [2].

For many years, bilirubin was considered only as a waste end product of heme catabolism – useless at best and toxic at worst. During the last few decades, however, a number of intriguing biochemical properties of bilirubin have been discovered, and there is now strong evidence for the beneficial role that bilirubin plays in the body, particularly as an antioxidant. There has also been a long history in Chinese traditional medicine of the beneficial health properties of ox gallstones, which consist largely of calcium bilirubinate.

IN VITRO STUDIES

Most of the interest in bilirubin as a potential therapeutic lies in its antioxidant properties. Bilirubin is probably the most abundant endogenous antioxidant in mammalian tissues [3]. The *in vitro* antioxidant properties of bilirubin were delineated largely by Stocker and coworkers in the late 1980s. He found that bilirubin, at micromolar concentrations, efficiently scavenged peroxyl radicals, either in homogeneous solutions or in multilamellar liposomes, to a greater extent than α-tocopherol (vitamin E), which was considered at the time to be the best antioxidant of lipid peroxidation [4]. In further studies, Stocker and Ames [5] showed that a water-soluble bilirubin-taurine conjugate could prevent radical-induced oxidation of phosphatidylcholine in either micelles or multilamellar liposomes, and that the same conjugate greatly accelerated Cu^{2+}-catalysed decomposition of linoleic acid hydroperoxide. As well as describing the antioxidant effects of this bilirubin conjugate, these workers also showed that albumin-bound bilirubin, at concentrations comparable to those present in normal plasma, also had antioxidant activity, and was capable of protecting albumin-bound linoleic acid against radical-induced oxidation. In competition studies, albumin-conjugated bilirubin was also found to out-compete an equimolar concentration of uric acid for peroxyl radicals, but was less efficient in scavenging these radicals than was ascorbic acid [6]. In later work, Stocker and Ernst [7] demonstrated the synergistic interaction between bilirubin and vitamin E in inhibiting the oxidation of phosphatidylcholine liposomes. Low micromolar

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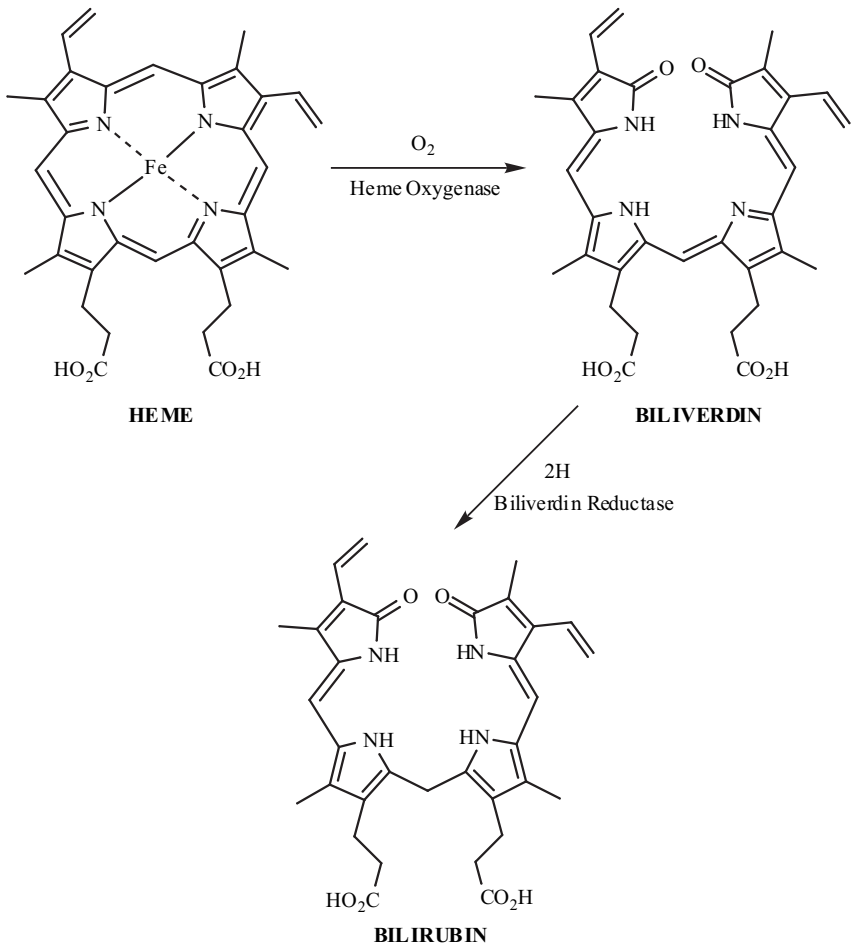

Fig. (1). Metabolic pathway from heme to bilirubin.

concentrations of bilirubin were able to inhibit oxidation of these liposomes in a concentration-dependent manner, unlike ascorbic acid or glutathione, which were ineffective. These authors also showed that low micromolar concentrations of bilirubin at physiological pH could efficiently scavenge hypochlorous acid [8].

Frei and coworkers monitored the depletion of endogenous ascorbate, thiols and bilirubin in plasma after exposure to aqueous peroxyl radicals, and they found that bilirubin was more effective at protecting lipids from peroxidative damage than other endogenous antioxidants [9]. The ability of bilirubin to scavenge superoxide radical has also been investigated, with bilirubin being approximately equal in activity with serum albumin, more active than the water-soluble vitamin E analogue Trolox, but less active than ascorbic acid [10]. In contrast, bilirubin has been shown to protect serum albumin itself against oxidation by hydroxyl radicals, to a greater extent than either ascorbic acid or Trolox [11]. More recently, bilirubin has been shown to act as an antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma [12].

Wu *et al.* have studied the protective effects of bilirubin against oxidation of human low-density lipoprotein (LDL). Oxidation of LDL is implicated in plaque formation in blood vessels leading to atherogenesis, and there is evidence that prevention of this oxidation reduces the incidence of coronary heart disease. Bilirubin at a concentration of 17 μM was found to protect against Cu^{2+}- mediated oxidation of LDL at least 20 times more effectively than Trolox [13].

Cahyana has noted that bilirubin has an antioxidant effect similar to that of the porphyrins [14], and Dailly has correlated bilirubin levels in plasma with the plasma's total peroxyl radical trapping activity [15]. More recently, Asad *et al.* have shown that bilirubin inhibits L-DOPA-Cu^{2+}-mediated DNA cleavage, and that bilirubin directly quenches the hydroxyl radicals generated by the L-DOPA-Cu^{2+} system [16].

Other known biological effects of bilirubin are its ability to inhibit the mutagenicity of 4-nitroquinoline *N*-oxide in strain TA100 of *Salmonella typhimurium* [17], and its ability to block the complement cascade, especially the C1 step [18].

CELLULAR STUDIES

Motterlini and coworkers showed that exogenously applied bilirubin could attenuate hydrogen peroxide-induced damage in vascular endothelial cells [19]. Later studies by Clark *et al.* showed that the addition of bilirubin to the culture medium of vascular smooth muscle cells could markedly reduce hydrogen peroxide-induced cytotoxicity.

These authors further found that hemin-mediated up-regulation of heme oxygenase led to increased levels of bilirubin, resulting in high resistance to cell injury caused by hydrogen peroxide, providing strong evidence that bilirubin generated after up-regulation of the heme oxygenase pathway is cytoprotective against oxidative stress [20]. Doré *et al.* have also shown that bilirubin conjugated to human serum albumin is neuroprotective, reversing the neurotoxic effects of hydrogen peroxide on hippocampal neuronal cultures, at concentrations as low as 10 nM [21]. Arai *et al.* have also shown that bilirubin's ability to scavenge reactive oxygen species impairs the bactericidal activity of neutrophils in a dose-dependent manner [22].

ANIMAL AND HUMAN STUDIES

Yamaguchi and coworkers have investigated the ischemia-reperfusion of rat liver and found evidence to suggest that bilirubin acts as an antioxidant *in vivo* under these conditions, and that bilirubin biosynthesis is increased by oxidative stress [23]. Other work by these authors with scurvy-prone ODS-*od/od* rats treated with lipopolysaccharide showed that bilirubin acts synergistically as an antioxidant with ascorbic acid [24]. Hyperbilirubinemia is commonly observed in newborn humans, and the possible protective role of bilirubin in neonates has long been debated. Evidence for the protective effects of bilirubin has been provided by Dennery *et al.*, who showed that bilirubin protects neonatal rats exposed to hyperoxia against serum oxidative damage in the first few days of life [25].

Clark *et al.* [26] have examined the effects of bilirubin on the protection of the rat heart against postischemic myocardial dysfunction. They found that treatment of the animals with hemin 24 h before ischemia reduced infarct size on reperfusion of isolated hearts. Exogenously administered bilirubin at concentrations as low as 100 nM significantly restored myocardial function and minimised both infarct size and damage to mitochondria on reperfusion, providing strong evidence of the cardioprotective effects of bilirubin against reperfusion injury.

Mildly increased serum bilirubin levels have been suggested to act as a protective factor, reducing the risk of coronary artery disease (CAD) in humans [27]. Hopkins *et al*. have tested this hypothesis on patients with early familial CAD and found that serum bilirubin was strongly and inversely related to CAD risk [28]. This work has been extended by Madhaven *et al.*, who found an inverse relationship between bilirubin levels and family history of heart decease. Also found were inverse relationships between bilirubin levels and both cigarette smoking and adiposity [29].

TOXICITY

Bilirubin commonly accumulates in the serum of neonates, particularly premature babies, causing hyperbilirubinemia (jaundice). At high concentrations, particularly in premature or low birth weight babies, bilirubin can deposit in the brain causing the neurotoxicity associated with kernicterus. Bilirubin has been shown to display some toxicity towards erythrocytes [30], and Amato has demonstrated a dose-dependent relationship between bilirubin levels and tyrosine uptake in rat synaptosomes, providing a plausible mechanism for bilirubin's neurotoxicity. Hansen and Allen [31] have reported that neurons are more sensitive to the toxic effects of bilirubin than are glial cells.

Hansen *et al.* have shown that bilirubin has widespread inhibitory effects on protein phosphorylation, inhibiting cAMP-dependent, cGMP-dependent, Ca^{2+}-calmodulin-dependent and Ca^{2+}-phospholipid-dependent protein kinases, with IC_{50}s ranging from 20 to 125 μM [32]. Amato also provided evidence that bilirubin can interfere with surfactant proteins at the air-liquid interface in the lung, with implications for the treatment of neonatal respiratory distress syndrome [33].

OX GALLSTONES

Traditional Chinese medicine prizes highly the medicinal properties of ox gallstones (also known as Niu Huang, calculus bovis, or bezoar). These gallstones are said to possess calming, antipyretic and antiinflammatory properties. The main constituent of ox gallstone is the calcium salt of bilirubin, with lesser amounts of cholic acid and deoxycholic acid [34]. There are a few reports of *in vitro* and *in vivo* examinations of the biological properties of these products. For example, Takahashi *et al.* have examined the effects of ox gallstone extract on the beating pattern of spontaneously contracting cultured embryonic mouse myocardial cell, and they showed that addition of the gallstone extract attenuated the cellular response to varying calcium concentration [35]. Rather than attributing this effect to bilirubin however, these authors suggested a possible role of taurine, identified in the gallstone extract, for the observed effects on the myocardial cells.

Antiviral activity of ox gallstone has been identified against encephalitis B *in vitro* and in mice. Bilirubin also showed *in vitro* activity, but not a high as ox gallstone. Antiviral activity was also observed in mice inoculated with the encephalitis virus, with the ox gallstone offering higher protection than bilirubin alone. The authors proposed a possible role for deoxycholic acid in the biological activities of the ox gallstone [36].

The effects of ox gallstone on the humoral immune response have also been examined in mice injected with sheep red blood cells. Short-term (1-2 days) daily oral dosage with ox gallstone stimulated the production of nitric oxide in mouse macrophages, whereas longer-term (7-14 days) daily oral dosage inhibited nitric oxide production, as well as decreasing TNF-α and IL-6 production in macrophages [37].

Li *et al.* have examined the antiinflammatory effects of artificial ox gallstone in mice and rats using both the croton oil-induced mouse ear edema, and the carrageenan-induced rat hind paw edema. Ox gallstone was shown to significantly inhibit edema in both species. The ability of ox

gallstone to inhibit the synthesis of nitric oxide was suggested to explain its antiinflammatory effects [38].

Finally, Nakashima *et al.* have investigated the effects of ox gallstone on rats given i.p. administration of carbon tetrachloride to induce liver toxicity. They found that oral administration of ox gallstone significantly increased both serum transaminase levels and hepatic lipid peroxidation, as well as increasing hepatic blood flow. This resulted in ox gallstone exacerbating carbon tetrachloride-induced hepatic damage through accelerated delivery to the liver from the peritoneal cavity [39].

SUMMARY

The reputation of bilirubin has been transformed from that of a toxin responsible for jaundice with no beneficial effects, to that of a biologically important antioxidant with a wide range of protective actions. Bilirubin is an effective radical scavenger at biologically relevant concentrations, more so than most other endogenous antioxidants. Several biological effects have been demonstrated in cells, including protective action against peroxide-induced damage, and neuroprotection of neuronal cultures against hydrogen peroxide damage. Antioxidant activity has also been demonstrated in animals, and it has been demonstrated that bilirubin biosynthesis is increased by oxidative stress. More recently the medicinal properties of ox gallstone (largely calcium bilirubinate) in rats have been examined, with interesting effects on the liver, on cytokine production and immune responses discovered.

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