Original Research Article

Bilirubin as savior of biological system

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Abstract

Bilirubin is more than just the final product of heme catabolism. Today it is considered to be a fundamental substance which acts as an antioxidant and anti-inflammatory agent in the serum. It can neutralize free radicals and prevent peroxidation of lipids. In addition there is evidence that it protects the cardiovascular system, neuronal systems, the hepatobiliary system, the pulmonary system and the immune system. Recently the use of pharmacological agents which augment expression of Heme oxigenase 1 (HO-1) has been investigated. Consequently its metabolites such as carbon monoxide (CO), biliverdin (BV) and bilirubin (BR) could become parts of a therapeutic strategy for treatment of various inflammatory illnesses. Reactive oxygen species (ROS) and signaling events are involved in the pathogenesis of endothelial dysfunction and represent a major contribution to vascular regulation. But depending on the amount of ROS production it might have toxic or protective effects. Despite a large number of negative outcomes in large clinical trials (e.g., HOPE, HOPE-TOO), antioxidant molecules and agents are important players to influence the critical balance between production and elimination of reactive oxygen and nitrogen species. With the present review we would like to highlight the important antioxidant role of the HO system and especially discuss the contribution of the biliverdin, bilirubin, and biliverdin reductase (BVR) to these beneficial effects.

Keywords: Biliverdin, bilirubin, antioxidant, anti-inflammatory, cytoprotection.

Introduction

Bilirubin (BR) has been commonly considered to be simply the "final product" of heme catabolism. Healthy newborns have high concentrations of it as a result of fetal erythrocytes breaking combined with its transitory inability to combine with glucuronic acid. In addition, high levels of it can end up accumulating inside the brain causing irreversible damage in areas such as the basal ganglia, producing kernicterus or bilirubin encephalopathy [1, 2]. However, for the past 20 years bilirubin has increasingly become known for physiological functions in normal concentrations. It as a powerful antioxidant and anti-inflammatory that can help prevent lipid oxidation and other kinds of oxidation with better efficiency than vitamin E [3, 4]. It has also been postulated as one of the principle defense mechanisms in the serum against oxidative stress [5, 6] and may have protective effects for the pulmonary, [7], cerebral [8], Hepatobiliary [9], immunological and cardiovascular systems [1, 3]. The concept of non conjugated bilirubin as a powerful antioxidant could be the answer, from a teleological point of view, to human necessities for producing bilirubin and not stopping the catabolic chain from heme to biliverdin (BV) like

amphibian, reptiles and birds do [10]. Biliverdin is a hydrosoluble substance that is easily excreted into the bile without spending an extra amount of energy or using other enzyme systems such as biliverdin reductase to finally produce bilirubin, a substance that is insoluble in water. Moreover, it needs albumin for transportation and for its conjugate excretion with glucuronic acid through UDP glucuronyl transferase 1 which makes it hydrosoluble, so it can be excreted into the bile and the small intestine [11]. This article will review evidence that supports the concept of bilirubin as a protective substance for human beings. Every day human beings produce 4 mg of bilirubin per kilogram of weight. The process starts with molecules that contain heme which is present in the hemoglobin of red blood cells and in other heme proteins such as cytochrome, catalases, peroxidases and tryptophan pyrrolase [11, 12]. 80% of the heme group comes from hemoglobin released by senescent erythrocytes and ineffective erythropoiesis. The other 20% is related to enzymatic non-erythroid sources [11, 12]. It has been proven that there are two bilirubin production peaks after an intravenous administration of porphyrin precursor radiation markers such as D aminolevulinic acid or glycine. One, due to ineffective erythropoiesis, occurs after 72 hours. The other, due to the destruction of senescent erythrocytes, occurs after 110 days [11]. Free heme is dangerous in excessive quantities [5] which is why it is quickly removed from tissues through hydrolyzation by microsomal heme-oxigenase, specifically at the -methane bridge.

This results in production of biliverdin, a carbon monoxide molecule (CO), the release of iron, and consumption of oxygen. The process requires a reducing agent, NADPH [6]. CO is a neurotransmitter and a powerful anti-inflammatory [7]. There are three different kinds of heme-oxigenase isoforms. The first one is isoform HO-1, inducible by stress or by the aforementioned heme. Isoforms HO-2 is a protein primarily made in the testicles and the brain. Isoforms HO-3 has very low catalytic activity. Its main function is as a linking protein to heme. HO-1 has a high concentration in the spleen and is responsible for heme quick elimination from circulation. It seems that HO-2 can protect neurons from oxidative damage. Heme oxigenase is part of the system which regulates the integrity of endothelial cells and which also regulates oxidative stress. The heme group of enzymes is very important for endothelial cells because it regulates activities of soluble guanylate cyclases (GCs), nitric oxide synthase (NOS), cytochrome P450 (CYP450), mono-oxigenase, cyclooxygenases (COX) and catalases [1, 2]. As will be discussed later, its induction or overexpression plays an important role in cellular lesions mediated by oxidative stress. HO-1 can be expressed not only by stimulating its free heme substrate, but also by diverse pro-inflammatory stimuli. This expands its functions from degrading heme groups to eliminating inflammation [2, 3] and to antioxidant and anti-inflammatory effects. This is mainly the result of biliverdin and bilirubin formation [4].
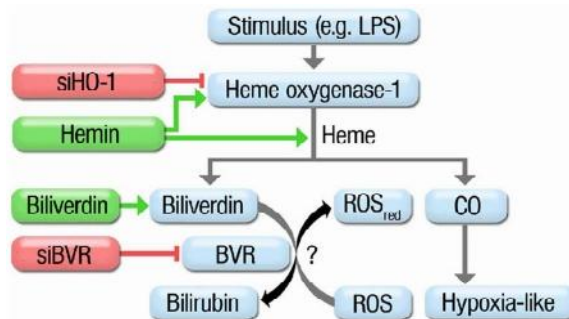

Figure-1 **Scheme summarizing the protective routes in the heme oxygenase-1 system.**

Review

The endothelial integrity is crucial for physiologic organ function and protects against vascular inflammation, sepsis, prothrombotic activity, and atherosclerosis (Bassenge et al., 2005). Disruption of this blood–tissue (endothelial) barrier results in endothelial dysfunction which is a hallmark of most cardiovascular complications (Cai and Harrison, 2000; Munzel et al., 2008). Reactive oxygen species (ROS) and signaling events are involved in the pathogenesis of endothelial dysfunction and represent a major contribution to vascular regulation. ROS are important regulators for cellular and metabolic conditions. Molecular signaling is highly dependent on ROS. But depending on the amount of ROS production it might have toxic or protective effects (Bachschmid et al., 2005; Daiber and Münzel, 2006; Ullrich and Kissner, 2006). Despite a large number of negative outcomes in large clinical trials (HOPE, HOPE-TOO as well as a prospective study with vitamin C in postmenopausal women with diabetes mellitus; Yusuf et al., 2000; Muntwyler et al., 2002; Lee et al., 2004; Mann et al., 2004; Lonn et al., 2005), antioxidant molecules and agents are important players to influence the critical balance between production and elimination of reactive oxygen and nitrogen species (RONS). The latter assumption is supported by a great number of experimental animal studies (Watanabe et al., 1997; Bassenge et al., 1998; Crawford et al., 1998; Elhaimeur et al., 2002) as well as human studies with rather small numbers of patients and acute infusion of antioxidants (Heitzer et al., 1996; Gori et al., 2001) indicating that antioxidants may be highly beneficial in improving endothelial dysfunction. The main reasons for the failure of chronic oral antioxidant therapy could be as follows: vitamin E and C act as pro-oxidants (e.g., tocopheryl and ascorbyl radicals), the coronary artery disease (CAD) of included patients is already irreversible, the CAD patients are already treated with drugs displaying antioxidant properties [e.g., angiotensin-converting enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARBs)], chronic antioxidant therapy inhibits intrinsic ischemic preconditioning which relies on RONS formation or oral vitamin treatment does not result in high enough concentrations of the antioxidants at the place of oxidative stress (Chen et al., 2012).Some of these reasons would favor the acute infusion of vitamin C in accordance with respective observations. However, infusion of antioxidants cannot be applied to a large number of patients over a long period and in addition may be affected by some of the draw backs listed above under chronic administration (e.g., interference with cellular redox signaling, suppression of oxidant-triggered ischemic preconditioning). Therefore, it may be a much more promising attempt to induce intrinsic antioxidant pathways in order to increase the antioxidants not systemically but at the place of oxidative stress and complications. A number of experimental animal studies have demonstrated that overexpression of ROS sources aggravates cardiovascular complications, whereas their suppression improves these adverse effects: the genetic deletion of NADPH oxidase subunits improved myocardial infarction damage and survival of mice (Doerries et al., 2007) and prevented angiotensin-II (Landmesser et al., 2002) as well as renovascular (clip model; Jung et al., 2004) induced hypertension, oxidative stress, and endothelial dysfunction. In contrast, overexpression of NADPH oxidase subunits further aggravated these complications (Dikalova et al., 2005). Vice versa, deletion of the mitochondrial manganese superoxide dismutase (MnSOD) increased age-dependent mitochondrial oxidative stress and endothelial dysfunction (Wenzel et al., 2008a) and deficiency in glutathione peroxidase-1 potentiated atherosclerosis and vascular complications in $ApoE^{-/-}$ mice (Torzewski et al., 2007). The recent observation that overexpression of mitochondrial superoxide dismutase improves angiotensin-II triggered hypertension and vascular dysfunction in mice shows that the different ROS sources can directly influence each other (Dikalova et al., 2010) and that especially the cross-talk between the mitochondrial and NADPH oxidase redox axis may play an important role for various diseases (Daiber, 2010).
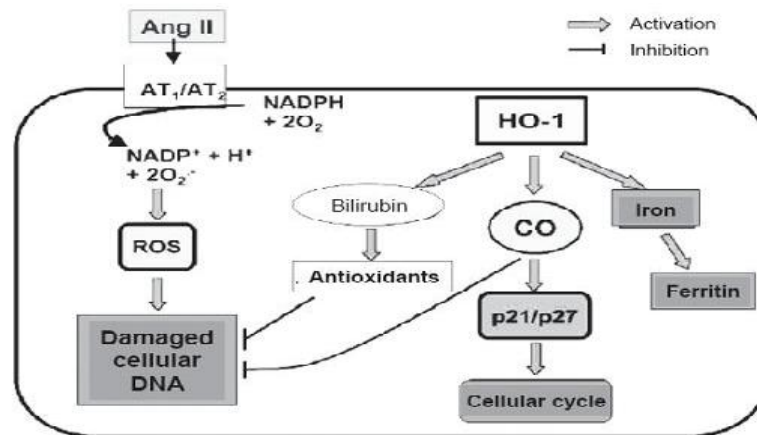
Fig.2 Hypothesis on OH-1 protective mechanisms to the angiotensin II induced lesion. Ang II: Angiotensin II. AT1/2: Receptor 1 and 2 of Angiotensin II. HO-1: Heme Oxigenase 1; CO carbon monoxide. ROS: Reactive Oxygen Species. Angiotensin II. ATI/ 2: Angiotensin II receptors 1 and 2. HO-1: Heme oxigenase 1; CO Carbon monoxide. ROS: Reactive Oxygen Species.

Observation

In a recent study of 55 families (33), 188 male and 144 female patients were randomized for several cardiovascular risk factors to determine if low bilirubin levels are related to early cardiovascular events (Early defined for men as before the age of 55, and for women before the age of 65 years). High albumen levels and low levels of high density lipoproteins (HDL) were related to high bilirubin levels in women but not in men. Low bilirubin levels were related to small increases in cardiovascular events in men, but not in women. Bilirubin gene secretion was found in 23% of the population. It was concluded that high bilirubin levels have a small effect on decreasing cardiovascular risk in men with no differences in women, possibly due to low HDL-C levels (33. Low bilirubin levels are also independent of, and inversely related to, the deterioration of carotid flow. This is mediated through vasodilatation and increased carotid artery intima-media thickness in both men and women, making these two factors predictors of atherosclerosis [5]. A correlation was also found between the highest bilirubin levels and lower risk of peripheral arterial disease. The National Health and Nutrition Examination Survey (NHANES), undertaken from 1999 to 2004 [6] analyzed 7075 patients   levels of total bilirubin and risk factors for peripheral arterial disease (EAP). It found, that a 0.1mg/dl bilirubin increment was associated with a 6% decrease in EAP. The odds ratio (OR) was 0.94. The 95% confidence interval was 0.9 to 0.98. Hyperbilirubinemia results were not due to chronic hepatic disease or alcohol consumption. An inverse relation was found between EAP and bilirubin levels in men with an OR of 0.90 and a 95% confidence interval between 0.85 and 0.96 while in women the OR was 0.97 and the 95% confidence interval was between 0.91 and 1.04. There was a B association between smokers whose OR was 0.81 with a 95% confidence interval between 0.73 and 0.90) and non-smokers whose OR was 0.97 and whose 95% confidence interval was between 0.93 and 1.02. The study results led to the conclusion that high bilirubin levels are associated with lower EAP prevalence. In a metaanalysis a negative association was also found between high bilirubin levels and severity of atherosclerosis (37). In addition to neutralizing oxygen radicals, unconjugated bilirubin also acts as a reducing agent of some peroxides including prostaglandin H synthase (PGH) when in the presence of hydrogen peroxide or organic hydroperoxides (38). Recently, Mazza and colleagues demonstrated bilirubin as antioxidant and cytoprotective effects in relation to damage to endothelial cells mediated by angiotensin II [3, 8]. Other authors have found that angiotensin II significantly stimulates peroxide formation in monocytes. Exogenous application of bilirubin

not only suppresses peroxide formation, but also suppresses the chemotactic activity of angiotensin II in these cells (39). Consequently bilirubin action can be particularly relevant to preventing vasoconstriction mediated by oxidation mechanisms. Within the mechanisms of action along this pathway it has been demonstrated that inhibiting NADPH oxidase and protein kinase C

(PKC) activity prevents vascular damage mediated angiotensin II [40]. Long ago has it been known that angiotensin II is frequently elevated in hypertensive people and associated to high levels of free radicals in oxygen which increase renal damage and therefore, natural antioxidants like bilirubin have protector effects (figure 3).
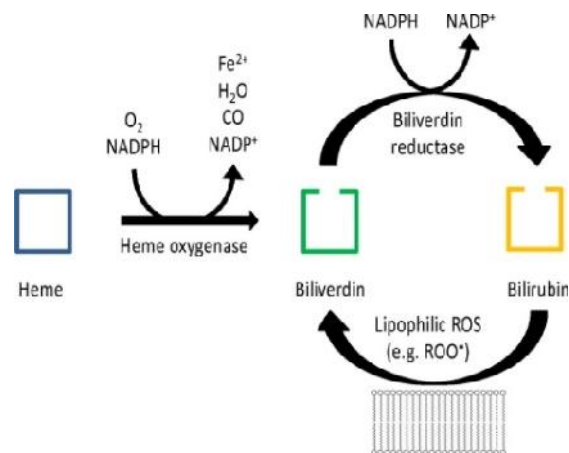

Fig.3 **Hypothetical scheme of the antioxidant redox cycle of the BR/BV/BVR system**

Results

As shown by Jansen et al. (2010) bilirubin represents a superior antioxidant as compared to biliverdin when applied in high concentrations. Using cell-free systems (test tube chemistry) the direct antioxidant effects of bilirubin versus biliverdin were assessed. The peroxynitrite scavenging ability of both bile pigments was assessed by two different biochemical models: tyrosine residues in bovine serum albumin (BSA) were either nitrated by authentic peroxynitrite ($ONOO^-$) or *in situ* generated $ONOO^-$ from the thermal decomposition of 3-morpholino-sydnonimine (Sin-1), a more physiological model to assess peroxynitrite-mediated oxidations. Peroxynitrite-mediated tyrosine nitration involves tyrosyl-radical-intermediates as a consequence of hemolytic bond cleavage in ONO-OH yielding hydroxyl ($HO^•$) and nitrogen dioxide ($^•NO_2$) radicals. Therefore, inhibition of peroxynitrite-mediated BSA nitration may be regarded as ability of BR and BV to scavenge peroxynitrite-derived free radicals and/or reduction of tyrosyl-radical-intermediates. According to our results, BR is at least threefold more potent than BV in

inhibiting peroxynitrite-mediated protein tyrosine nitration. The used dot blot technique excludes any interference of the BR or BV color since the compounds are removed during transfer of the sample to the membrane. Superoxide scavenging ability of BR and BV was determined in two different systems. In the first one superoxide was constantly generated by xanthine oxidase (XO) and hypoxanthine whereas in the second one we used authentic superoxide (KO_2) to exclude any inhibitory effects of the compounds on enzymatic activity (e.g., one of the compounds could be an inhibitor of XO). Since the color of BR and BV may lead to false-positive results using direct optical methods, we used HPLC-based detection of fluorescent 2-hydroxyethidium (2-HE), the specific oxidation product of dihydroethidium and superoxide. XO-derived superoxide was decreased in a concentration-dependent fashion by BR whereas BV had no inhibitory effect and even significantly increased the signal pointing toward pro-oxidative effects. It should be noted that the absolute increase in superoxide signal by BV was small (approximately 10%). Using KO_2 from a saturated stock solution in dimethyl sulfoxide (DMSO), the above described

differences even became more pronounced: BR decreased the formation of 2-hydroxyethidium in a concentration-dependent fashion whereas BV, this time, dramatically increased its yield up to 2.5-fold over KO_2 control without BR or BV. This may be taken as evidence for redox-cycling of BV amplifying the superoxide formation rate. Again, the used HPLC technique excludes any interference of the BR or BV color since the compounds are separated from the product during their way through the column. Previous reports suggested an efficient catalytic cycle between BV and BR (Baranano et al., 2002; Sedlak and Snyder, 2004; Liu et al., 2006) and others have observed conversion of BR to BV by peroxynitrite (Kaur et al., 2003), or peroxyl radicals (Baranano et al., 2002). In support of these previous findings and a role of BVR in cytoprotection, Young et al. (2009) demonstrated that angiotensin-II induced superoxide formation in renal tubular epithelial cells and inner medullary collecting duct (IMCD3) cells is aggravated by silencing of BVR. Superoxide formation was measured using dihydroethidine (DHE) fluorescence and lucigenin-derived chemiluminescence. BVR silencing resulted in a significantly decreased level of BVR protein (>30% of control cells) as well as diminished cellular bilirubin concentrations (50% of control cells). Co-treatment with BV could decrease the angiotensin-II induced superoxide formation in control cells, whereas it further aggravated the ROS levels in BVR-silenced cells. This is in good accordance with our observations on increased superoxide levels by biliverdin in the presence of xanthine oxidase or authentic superoxide, most probably via redox-cycling (see the proceeding chapter). However, it should be noted that Young et al. also observed BVR/BR independent antioxidant action of HO-1, since hemin co-treatment improved superoxide formation in BVR-silenced cells, indicating an important contribution of CO (and other so far not identified routes). The beneficial effect of hemin could of course also be related to increased break-down of superoxide in the presence of hemin leading to other reactive intermediates or non-toxic products that are not detected by DHE fluorescence.

Discussion

Arteriosclerosis is an inflammatory disease of the walls of large and medium size arteries which is precipitated by elevated levels of low density lipoproteins in the blood [5]. Oxidative modifications in the plasma of low density lipoproteins (LDL), mainly at the level of arterial walls, markedly increases the atherogenicity of these [5, 3] and, with the deposit of oxidized LDL, form part of the crucial lesions of arteriosclerosis [5, 4]. Minimally modified LDL induces chemotactic protein-1 monocytes and colony-stimulating factor which produce recruitment and differentiation of macrophages in the arterial wall (54). The endothelial cell dysfunction induced by oxidized LDL is one of the first steps in the development of arteriosclerosis, so the adaptive vascular responses and /or oxidative stress protectors are important in the prevention of arteriogenesis [3]. Inhibition of LDL oxidation is one of the anti-arteriogenic properties of bilirubin. This includes neutralizing free oxygen radicals generated from phospholipids, triglycerides and cholesterol esters [1, 6]. Even at low concentrations it has the capacity to inactivate oxygen radicals *in Vitro*, decreasing oxidative cell damage and attenuation of oxidative stress *in vivo* (. The mechanisms by which bilirubin reacts with oxygen radicals are not completely understood, however its hydrophobic tetrapyrrole structure has been reported to be an inhibitor of NADPH oxidase and protein kinase C (PKC) among other mediators of pro-arteriogenic factors. Experimentally, bilirubin has immunosupresor effects which act on lymphocytes and granulocytes, [1]. In vitro, bilirubin in concentrations from 100 to 200 micromoles inhibits cytotoxic T lymphocyte activity [2]. Similarly, it alters the proliferation of T cells induced by phytohemagglutinin [3]. HO 1 has important immunosupresor effects and its induction decreases episodes of acute and chronic rejection in solid organ transplants [4].

Conclusions

In spite of the evidence of the antioxidant effect of bilirubin it is important to highlight that this effect basically occurs in the serum while the most important protective mechanisms are within

the tissues. Since bilirubin concentration is 100 to 1000 times greater in the serum than within cells, we still lack an elucidation of the interrelation of bilirubin activities within the serum and its activities which occur inside the cells [1]. The great challenge involved in understanding these physiological events is to apply what we learn to therapeutic applications. Recently, the results of biliverdin and bilirubin as therapeutic application in solid organ transplants have been checked [1,]. The use of pharmacological agents that increase HO-1 expression, and therefore its metabolites (carbon monoxide, biliverdin and bilirubin) can become a therapeutic strategy for handling different inflammatory diseases (24). This last possibility acquires greater relevance if we take into account the fact that the only currently available strategy is the use of immunosuppresor which increase the incidence of tumors and infectious bacterial, fungal and viral diseases in patients with transplants. Similarly, HO-1 is being considered as a potential therapeutic target for hepatoprotection [9]. In figure 4, the beneficial effects of the metabolites involved in the metabolic chain of heme metabolism (CO, BV/BR) are shown.

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