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Opinion

A Novel Perspective on the Biology of Bilirubin in Health and Disease

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Unconjugated bilirubin (UCB) is known to be one of the most potent endogenous antioxidant substances. While hyperbilirubinemia has long been recognized as an ominous sign of liver dysfunction, recent data strongly indicate that mildly elevated bilirubin (BLB) levels can be protective against an array of diseases associated with increased oxidative stress. These clinical observations are supported by new discoveries relating to the role of BLB in immunosuppression and inhibition of protein phosphorylation, resulting in the modulation of intracellular signaling pathways in vascular biology and cancer, among others. Collectively, the evidence suggests that targeting BLB metabolism could be considered a potential therapeutic approach to ameliorate a variety of conditions.

From a Biological Waste Product to a Potent Biological Compound

UCB (see Glossary), the end product of the heme catabolic pathway, has long been recognized as a sign of liver dysfunction or a potential toxic factor causing severe brain damage in newborns. Mildly elevated BLB levels, as seen in patients with **Gilbert syndrome**, have been shown to be protective against an array of diseases associated with increased oxidative stress, such as cardiovascular diseases (CVD), diabetes and cancer [1,2]. These clinical observations are consistent with recent discoveries relating to how BLB might affect the pathophysiology of these diseases (Box 1). BLB is recognized as the most potent endogenous antioxidant due to its continuous recovery in the **BLB/biliverdin (BLV) redox cycle** (Figure 1), resulting in protective lipid peroxidation both *in vitro* [HEK293 cells in which biliverdin reductase (BLVR) was silenced]

Box 1. The Clinician's Corner

Until recently, BLB was considered as a waste product of heme with very limited biological activity. However, BLB has been described as a sign of liver disorders since Hippocrates. More recently, evidence suggests that BLB and related products in BLB metabolism (the yellow players) have a major role in several cellular events.

Together with uric acid, BLB is one of the most active antioxidant molecules in the human body. This property may explain some of its effects on cellular functions (growth, migration, differentiation, and modulation of the immune response).

When translated into humans, these activities may account for the reduced prevalence of CVD diseases, cancer, and metabolic syndrome in subjects with slightly higher serum BLB levels, as is the case of patients with Gilbert syndrome. Less clear and still under study is the possible relation between serum BLB levels and neurological diseases.

On these grounds, the pharmacological modulation of the yellow players (mainly resulting in increasing UCB intracellular concentration) may become a promising and effective therapeutic approach.

Trends

Historically known for its toxicity but recently recognized as a powerful protective molecule, BLB is gaining more attention due to its pleiotropic biomolecular effects and those of the enzymes involved in BLB metabolism (the 'Yellow Players').

Both heme oxygenase (HMOX) and biliverdin reductase (BLVR) (the main enzymes in BLB metabolism) act on numerous signaling pathways, with unsuspected biological consequences. The interconnections of such pathways highlight an incredibly complex biomolecular network. Yellow player molecules can have important physiological and pathological biological outcomes. Their still unexplored roles merit attention, offering the possibility of being targeted for therapeutic benefit.

The moderately high levels of UCB in the blood of patients with Gilbert syndrome are suggestive of the protective role of BLB in non-neurological pathologies (cardiovascular diseases, cancer, and metabolic syndrome).

Cells and tissues might actively maintain the intracellular homeostasis of BLB, with the yellow players being viewed as novel antioxidant mechanisms in a cell.

This new point of view might also be applicable to neurological diseases, where BLB levels are lower than in healthy subjects.

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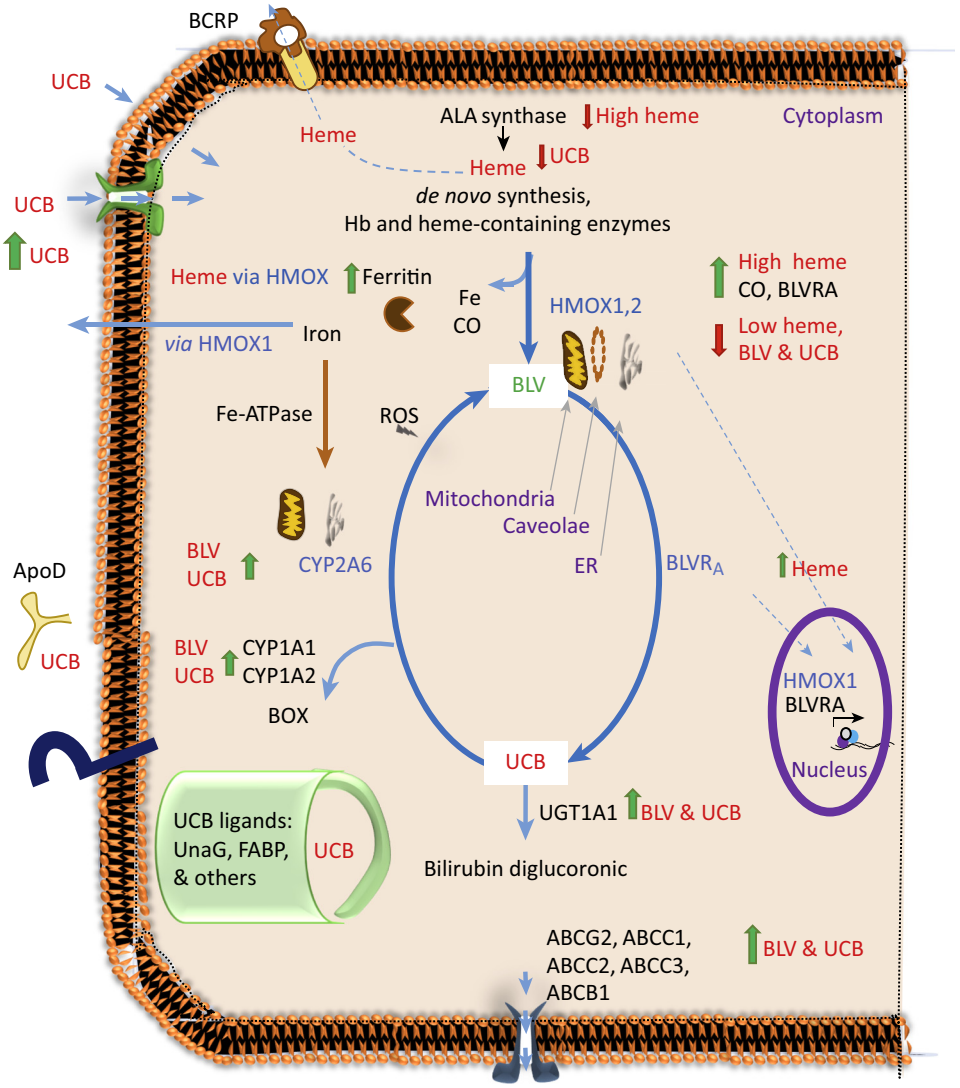

Trends in Molecular Medicine

Figure 1. Intracellular Bilirubin (BLB) Metabolism. Heme (produced by the action of the ALA: alanine synthase) [5-aminolevulinic acid (ALA)] is converted to biliverdin (BLV, plus CO, plus iron) [carbon monoxide (CO) (Fe)]by heme oxygenase (HMOX; localized in mitochondria, endoplasmic reticulum, and/or caveolae) 1 (inducible) and 2 (constitutive) enzymes. BLV is then converted to unconjugated bilirubin (UCB) by biliverdin reductase (BLVR) A. Thus, UCB may be produced either endogenously within the majority of the cells, or transported to the cell (typically hepatocytes): (i) by passive diffusion across the cellular bilayer due to its lipophilicity; or (ii) by apolipoprotein D (ApoD) expressed on the cell membraneor (iithe. BBLBalso UCB is stored inside the cell, bound to either (i) UnaG (belonging to the family of fatty acid-binding proteins, FABP); (ii) FABP1 (protein Z); (iii) lipids of the cellular membranes; or (iv) ligandin (glutathione S-transferase B = protein Y). UCB might be converted back to BLV by the activity of the cytochrome P450 mono-oxygenase 2A6 (CYP2A6), or during its oxidation by reactive oxygen species (ROS; sacrificial anode action). The intracellular UCB level might be reduced by either: (i) conjugation with glucuronic acid by the uridine-diphosphate glucuronosyl transferase 1A1 (UGT1A1), followed by (ii) efflux by ATP binding cassette (ABC) transporters G2, C2, C1, and B1; or (iii) oxidation by CYP1A1 and 1A2 (CYPs: localized in the mitochondria and/or endoplasmic reticulum) to bilirubin oxidation products (BOXes). Finally, the intracellular UCB level may be also regulated by export of heme, its precursor, out of the cell via breast cancer resistance protein (BCRP). All the enzymes involved in the UCB–BLV cycle are strictly interconnected and modulated (red arrows, inhibition; green arrows, induction). Both HMOX and BLVR might migrate into the nucleus and act as transcription factors.

and *in vivo* [heme oxygenase (HMOX) 2 knockout versus wild-type mice] [3]. BLB also exerts immunosuppressive effects on antigen-presenting cells [4] and T cells [5], as well as in the inhibition of adhesion molecule expression [6] and immune cell migration [7] (see below). Moreover, BLB exerts widespread inhibitory effects on protein phosphorylation, resulting in the substantial modulation of intracellular signaling pathways with multiple implications in vascular and autoimmune pathologies, as well as in cancer. Indeed, BLB has been shown to inhibit neointimal and vascular smooth muscle cell hyperplasia *in vivo* and *in vitro* [8], in addition to arresting tumor cell growth, possibly inducing apoptosis [9]. These concepts provide the basis for a new understanding of BLB metabolism, raising the possibility that modulating the levels and/or activities of serum BLB, HMOX, and BLVR (the **'yellow players')** could be a novel therapeutic tool to ameliorate atherosclerosis, cancer, autoimmunity, and/or neurodegenerative conditions. As we proceed in understanding the multiple roles of these yellow players in modulating various cellular pathways, we suggest using the term 'bilirubinomics' to describe this field of study.

A New Perspective on the Bilirubin–Biliverdin Antioxidant Cellular Cycle

Until recently, intracellular BLB was mainly considered to either derive from the blood or be regenerated from BLV via the BLV/BLB cycle [10]. The cycle is initiated by the microsomal HMOX1/2 (HMOX1 is inducible, whereas HMOX2 is constitutive) originating from BLV, and continued by the cytosolic BLV reductases (BLVRA and BLVRB) (Figure 1). The BLB antioxidant system involves *de novo* synthesis of heme mediated by the rate-limiting enzyme 5-amino-levulinic acid (ALA) synthase. The beneficial effect of ALA is well known in plant biology, and ALA is a potent supplement in commercial fertilizers to increase plant tolerance to environmental stress. In fact, protective effects of ALA against increased oxidative stress have been demonstrated *in vivo* in a mouse model of chronic hypoxia-induced pulmonary hypertension [11]. In this study, sufficient intracellular heme concentration in pulmonary cells was not only required for the production of heme proteins involved in an array of biological functions, such as mitochondrial superoxide production or nitric oxide (NO) generation, but was also an important stimulus for HMOX1 induction, one of the major antioxidant enzymes [12]. Indeed, clear therapeutic effects of hemin itself have been reported for several experimental oxidative stress-mediated as well as metabolic diseases, such as arterial hypertension [13] and diabetes [14] in rat experimental models. More recently, *de novo* intracellular synthesis of heme, which is then transformed into UCB [15], appears to have a crucial role in the defense against oxidative damage in human cell lines, including epidermal, kidney, hepatic, breast, colon, and erythroleukemia cancer cells [15]. A similar cytoprotective role of BLB was also noted *in vivo* based on the observation that UCB binds the **fatty acid-binding protein (FABP) UnaG** in the muscles of freshwater eels [16]. The resulting UnaG–UCB complex has been interpreted as an attempt to preserve and store this antioxidant to manage oxidative muscle metabolism during long-distance migration [16]. We speculate that BLB is also likely to have an important role in **phylogenesis**, because there is evidence for the widespread occurrence of FABPs in nature, with it being present across several vertebrate orders [17].

As for the BLB–BLV redox cycle, the mitochondrial **cytochrome P450 monooxygenase 2A6 isoform** (CYP 2A6) is also able to oxidize UCB back to BLV, as shown in yeast transfected with the human enzyme [18]); thus, CYP2A6 appears to be the 'missing enzyme' needed to complete the BLV-UCB cycle. It is not surprising that this enzymatic system operates to maintain intracellular BLB homeostasis, fine-tuning the 'yellow players' (Figure 2) with biological consequences that were unsuspected until recently.

As shown in mouse liver, the BLB-metabolizing enzymes are sequestrated within the cell in a highly integrated way, distinguishing between mitochondrial and cytoplasmic compartments (Figure 1) [19]. According to the current concept, the fate of intracellular BLB depends on the

Glossary

Aryl hydrocarbon receptor (AhR): ligand-activated transcription factor acting on aryl hydrocarbon response element (AHRE), xenobiotic response element (XRE), and drug response element (DRE) consensus regulatory sequences in the promoters of *HMOX1*, *CYP1A1/2*, *CYP2A6*, *UGT1A1*, *SLCO1B1* (encoding OATP2), and *ABCs*, which are involved in bile pigment metabolism and transport.

Bilirubin/biliverdin redox cycle: BLB may be converted back to BLV via its oxidation by reactive oxygen species present during pro-oxidant conditions. Regenerated BLV is then reduced back to BLB by BLBR. This redox cycle allows nanomolar concentrations of BLB to counteract millimolar concentrations of pro-oxidants in a cell.

Bilirubinomics: here, the large-scale study of multiple biological phenomena related to the molecular effect of UCB inside the cell, including DNA, RNA, proteins, and other macromolecules involved in genetic modulation and function.

Cytochrome P450 monooxygenases 2A6 isoforms (mouse 2a5): an enzyme present in the endoplasmic reticulum and/or mitochondria, demonstrated to enzymatically convert BLB back to BLV.

Fatty acid binding protein (FABP) UnaG: binds and transfers fatty acids and other lipophilic molecules from different cellular compartments. Specifically, UnaG (from *nihon unagi, Anguilla japonica*) was recently identified in the muscles of migrating fishes (eels, salmon, etc.) exposed to oxidative stress from prolonged muscle activity during migration. UnaG binds UCB, protecting it from oxidation, which is a possible mechanism to preserve and store UCB, thereby tackling oxidative stress in fish.

Gilbert syndrome: genetic disorder of BLB metabolism, resulting in less-efficient hepatic UCB conjugation due to mutations in the *UGT1A1* gene promoter. This results in slightly elevated and mild, fluctuating serum UCB levels and jaundiced individuals. The syndrome has been negatively correlated with risks of CVD, cancer, diabetes, and various metabolic conditions. The protective action of UCB has been attributed to its

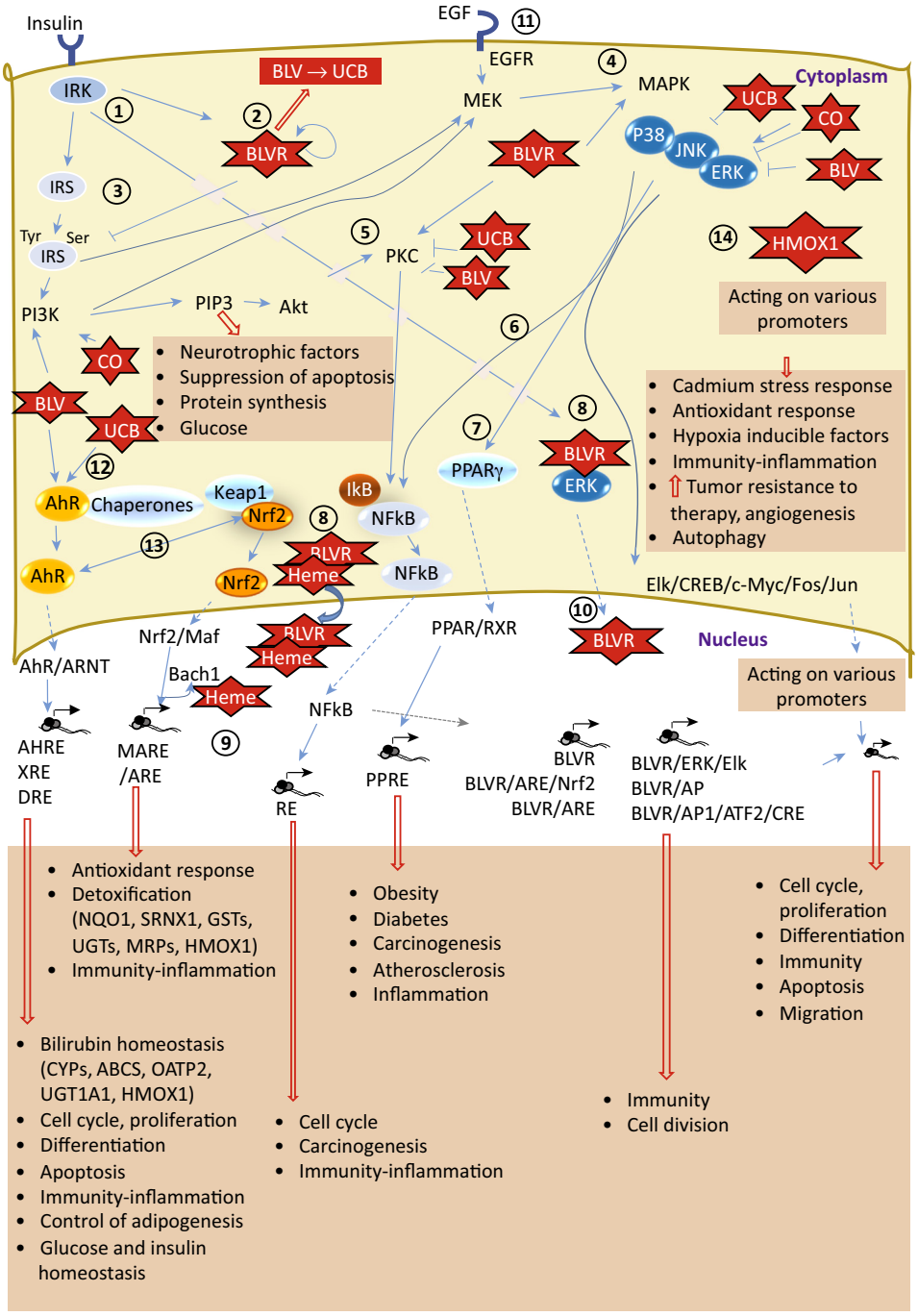

Figure 2. Interconnections and Biological Functions of the Yellow Players in the Cell. Cytoplasmic biliverdin reductase (BLVR) may: (1) be a substrate for the insulin receptor tyrosine kinase (IRK); (2) act as a kinase on itself; (3) inhibit [serine (Ser) phosphorylation] the insulin receptor substrate (IRS) pathway [phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), phosphatidylinositol (3,4,5)-trisphosphate (PIP3), protein kinase B (Akt), protein kinase C (PKC)] while IRK activates phosphorylating tyrosine (Tyr) residues; (4) act as a kinase on mitogen-activated protein kinases (MAPKs), p38, cJNK (Jun N-terminal kinases), ERK (extracellular signal-regulated kinases (ERK)]) pathway [passing through Elk (ETS domain-containing protein), CREB (cAMP response element-binding protein)/c-Myc/c-Fos/Jun/ATF (activating transcription factor 2)/AP (activacting protein)/ERG1 (ETS-related gene)/CRE (cAMP response element)]; and (5) on PKC, activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. (6) Alternatively, NF-κB might be activated via the

(Figure legend continued on the bottom of the next page.)

antioxidant, antiproliferative and immunomodulatory actions.

Hemin: oxidized form of heme, used as a potent inducer of HMOX1.

J series prostaglandins (PGs): Lipid-based hormones that interact with PPARγ, modulating proliferation, apoptosis and tumorigenesis. J PGs induce HMOX1 to presumably exert a protective effect from oxidative stress in cells.

Long-term potentiation (LTP) and long-term depression (LTD): activity to produce better and longer synaptic signals (LTP) or decreased synaptic signals (LTD).

The yellow players: the enzymes HMOX, BLVR, and CYP2A6 are involved in the production and recycling of BLB. Their coordinated activity can tightly control the amount of UCB, and they may be important in various cellular functions.

Transmembrane transporters: proteins regulating the passage of molecules, ions, and solutes across the cellular bilayer; these include MRP/ABCs transporters, BCRP, and OATPs.

UCB/UnaG in phylogenesis: the widespread occurrence of FABPs binding UCB within cells across several vertebrate orders; such binding could represent an evolutionary advantage to protect organisms against increased oxidative stress.

UGT1A1: enzyme catalyzing the addition of one or two glucuronic acid residues to the lipophilic UCB, transforming it into a water-soluble (extractable) conjugated BLB. Mutations affecting the gene lead to increased UCB levels in serum and tissues.

Unconjugated bilirubin (UCB): product of the heme catabolic pathway, by the chain action of HMOX converting heme to BLV, and BLVR converting BLV into UCB; UCB is a powerful antioxidant molecule.

degree of oxidative stress. This has been shown in mouse hepatic tissue, where the equilibrium is regulated by mitochondrial Cyp2A5 (the mouse ortholog of CYP2A6) preventing an excess of intracellular BLB [19].

Other mechanisms are also likely to have an important role in intracellular BLB homeostasis. Examples of these include the glucuronosylation of BLB by the enzyme **uridine diphospho-glucoronosyl transferase 1A1** (**UGT1A1)** [19], resulting in increased BLB water solubility and allowing its excretion in bile. Alternatively, cellular BLB flux is regulated by the ATP-binding cassette transporters ABCC1/2/3, ABCG2, and ABCB1, together with the organic anion transporting polypeptide (OATP), which is involved in the regulation of the intracellular levels of heme (Figure 1).

Both UCB and BLV exert regulatory functions in multiple biological processes (Figure 2), and are potent endogenous activators of the **aryl hydrocarbon receptor (AhR)**, a ligand-activated transcription factor acting on various genes, including *HMOX1* [20], *CYP1A1/2*, *CYP2A6*, *UGT1A1* [21], *SLCO1B1* (encoding OATP2) [22], and *ABC*s, involved in BLB biotransformation and transport. Indeed, the AhR signaling pathway appears to have a wider impact, since it is known to be part of a complex network including cell cycle regulation, mitogen-activated protein kinase (MAPK) cascade activation, and nuclear factor-erythroid-2-like 2 signaling [encoded by *NFE2L2* (also known as *Nrf2*)]. These pathways induce a battery of genes linked to AhR/Nrf2 signaling [23], and the biological implications of this are illustrated in Figure 2. The modulatory role of AhR in kinase reactions may account for the potent inhibitory effects of UCB on protein phosphorylation, although this has not yet been extensively investigated. As shown in Figure 2, target genes include those involved in apoptosis, T helper-mediated immune responses [24,25], and cellular proliferation and differentiation (vascular endothelial cells, smooth muscle cells, and macrophages), with important implications in carcinogenesis [20]. Although AhR activation may induce proliferation, stimulation by endogenous substrates, such as UCB, may mediate cell cycle arrest, as demonstrated in LoVo human colon cancer cells *in vitro*, where AhR activation was shown to inhibit cell proliferation, inducing G1 cell cycle arrest via *the* downregulation of cyclin D1 and Rb protein phosphorylation [26]. Similar results were previously observed in rat mammary and human pancreatic cancer cells [27]. Thus, AhR-mediated mechanisms may contribute to the apparent anticancer effects of BLB, reported in the Third National Health and Nutrition Examination Survey of more than 176 million subjects [28]. AhR *per se* is regulated by Nrf2, further strengthening the regulatory interplay between both factors (Figure 2, point 13) [29]. In addition, AhR has been reported to have a role in immune responses, regulating T regulatory (Treg) and T helper 17 (Th17) cell differentiation [25,30].

MAPK cascade. (7) MAPKs act also on peroxisome proliferator-activated receptor (PPAR)-γ, migrating thereafter into the nucleus and acting on *RXR* (pregnance X receptor)/*PPRE* (peroxisome proliferator-activated responsive element)-responsive genes. (8) BLVR itself may migrate into the nuclear compartment, transporting ERK and heme. (9) Heme acts by stabilizing Bach1 and inducing Nrf2 [nuclear factor (erythroid-derived 2)-like 2]-mediated biomolecular events [through Maf/ARE (antioxidant responsive elements)/MARE (Maf antioxidant responsive elements]. (10) Inside the nucleus, BLVR can act as a transcription factor, binding directly to ARE/AP1-2, and ATF2/CRE DNA sequences (present also on the *HMOX1* promoter–see point 14), or in complex with ERK/Elk (belonging to MAPK signaling pathway–see point 4), or Nrf2/ARE (belonging to the Nrf2 signaling pathway–see point 9). (11) IRK and MAPK pathways are interconnected through MEK (mitogen-activated protein kinase kinase), via the epidermal growth factor (EGF) signaling cascade. Several signaling pathways also respond to cellular unconjugated bilirubin (UCB), biliverdin (BLV), and carbon monoxide (CO) levels (arrow headed line, induction; bar headed line, inhibition), such as (12) aryl hydrocarbon receptor (AhR) translocation into the nucleus, interacting with ARNT (AhR nuclear translocator)/AHRE (Ahr responsive elements)/XRE (xenobiotic responsive element)/DRE (drug responsive element). (13) AhR and Nrf2 pathways are interconnected. (14) HMOX1 has a wide spectrum of DNA-binding motifs on its promoter [CRE/Erg1 (estrogen regulated gene)/NF-κB/AP2/HNE1,4 (4-hydroxynonenal)/HSF (heat shock factor)/Hif (hypoxia inducible factor)/cJun/Fos/AT,F and stRE (stress responsive elements), similar to the ARE/MARE binding site for Nrf2]. Abbreviations: Keap1, Kelch-like ECH-associated protein 1; GSTs, glutathione S-transferase; SRNX1, sulfiredoxin 1; NAD(P)H, quinone oxidoreductase 1; RE, responsive elements.

The HMOX and BLVR enzymes are expressed in a range of tissues and are synergistically induced by multiple stimuli provoking oxidative stress. In addition to producing UCB, BLVRA has several other biologically important actions [31] (Figure 2), including the unique multispecific (serine/threonine/tyrosine) kinase activity that contributes to cell signaling, as indicated from studies on human embryonic kidney cells transfected with *hBLVR* [32]. BLVRA, as well as HMOX1, can translocate from the cytosol into the nucleus, activating, in an oxidative stress-induced manner, transcription in a variety of signaling pathways (Figure 2), including those involving survival, the stress response, Jak-Stat [33], transforming growth factor (TGF)-β, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and p38 MAPK [34], as well as modulating the expression of HMOX itself and AP-2-regulated genes [35]. BLV and BLVRA have also been shown to modulate protein kinase C (PKC), a Ser/Thr kinase implicated in carcinogenesis [36]. This complex network suggests that intracellular UCB should be considered a part of the antioxidant cellular system, via which cells can modulate their content and functions.

Thus, it is reasonable to assume that each cell and/or tissue may have different thresholds of intracellular UCB concentrations that result in either protective or dangerous outcomes. Indeed, different amounts of UCB have been quantified in both physiological and pathological conditions in animal tissues [37], and different levels of toxicity have been reported for different cells.

Bilirubin in Cardiovascular Disease, Inflammatory Metabolic Syndrome, and Diabetes

In humans, a low (<7 μmol/l) total BLB concentration has been shown to be a risk factor for systemic diseases associated with increased oxidative stress, such as cardiovascular diseases (CVD), diabetes, metabolic syndrome, certain cancers, and autoimmune and neuropsychiatric diseases (reviewed in [38]). A meta-analysis study performed on a large male population with CVD showed that each micromolar decrease in serum BLB significantly increased the risk of atherosclerotic diseases [1], with a BLB concentration of 10 μmol/l being defined as the discriminating cut-off value. Compared with a BLB concentration > 10 μmol/l, a serum BLB concentration < 7 μmol/l was reported to increase the risk of CVD in the general population by 30%, comparable with that of high-density lipoprotein cholesterol [39], resulting in a new proposed cardiovascular risk calculation algorithm that includes the BLB concentration [40].

Recent studies on patients with colorectal cancer and Crohn's disease suggested a role of serum BLB as a global defense molecule against increased oxidative stress [41,42]. These clinical observations were supported by an *in vivo* model of inflammatory colitis in mice, where BLB concentrations were found to prevent injury [7]. Based on migration studies in T cell lines, it was suggested that vascular cell adhesion molecule 1 (VCAM-1)-mediated immune cell migration processes contributed to ameliorating the disease [7].

BLB acts not only against oxidative stress. The protective effects of mild hyperbilirubinemia are complex, affecting multiple stages of cell and tissue biology, as evidenced by both clinical and experimental studies. These have included reducing the effects of lipids on body weight in overweight and obese human subjects [43], blood pressure in hypertension (each micromolar increase in serum BLB decreased systolic blood pressure by 0.13 mm Hg [44]), and serum homocysteine concentrations in diabetic retinopathy [45]. BLB has also been reported to have immunomodulatory and anti-inflammatory effects [46], to modulate platelet functions and hemostasis [47], to influence vascular dysfunction, cell–cell adhesion [6], NO production in HUVEC and H5V cells [48], and intracellular signaling upon vascular injury in rats [8].

The progress in advancing our knowledge of the molecular mechanisms of BLB action is exemplified by the protective effect of BLB in diabetes and metabolic syndrome. Insulin-like

activities of BLB were reported in rat fat cells as early as 1980, and were recently confirmed by the observations that BLB can increase insulin sensitivity, ameliorate obesity, and suppress chronic inflammation and endoplasmic reticulum stress in leptin receptor-deficient (db/db) and diet-induced obese mice (DIO) [49]. BLB also exerts beneficial effects in DIO mice by reducing leptin, glycemia, and cholesterol concentrations, and by increasing adiponectin [50].

In obese mice, increased production of BLB activates peroxisome proliferator-activated receptor (PPAR)-α, fibroblast growth factor (FGF)-21 and glucose transporter (Glut)-1, resulting in reduced lipid droplet size, fatty acid synthase levels, body weight, and blood glucose [51]. These effects were confirmed in a PPARα-knockout mouse model [52]. Interestingly, the activating effects of PPARα on bile pigments were of the same magnitude as those of fenofibrate, a potent and clinically used activator of this nuclear receptor, and a recent *in silico* analysis revealed that PPARα ligands bear close structural similarities to BLB [52]. Of note, BLV enhances the expression of CD36 [50], which is involved in fatty acid oxidation and control of diabetes [53], and BLB increases the expression of PPARγ [50], another master regulator of adipogenesis and obesity [54]. The effects of BLB on AhR and PPARα-induced expression of FGF21 [55], a systemic insulin sensitizer, suggest that BLB itself harbors this property, which may account for the lower incidence of diabetes mellitus and metabolic syndrome in patients with Gilbert syndrome [38]. These mechanisms are likely to be implicated in conditions where HMOX1 is induced [51].

Based on the agonist effects of BLB on PPARγ, BLB metabolism appears to be interweaved with bile acid metabolism [56]. The link between both pathways might be PPARγ, since activation of this nuclear receptor in the intestine modulates bile acid metabolism via the FGF15/19 pathway [57].

Finally, heme is a strong modulator of adipogenesis, promoting the differentiation of fibroblasts to adipocytes in mouse 3T3-F442A cells; in line with this, it was demonstrated that increased heme catabolism by HMOX1 induction could increase adipogenesis in obesity and metabolic syndrome [58].

Bilirubin in Neurological Diseases

Clinical evidence indicates that lower serum BLB levels occur in a range of neurological diseases, such as Alzheimer disease (AD), dementia, multiple sclerosis, and cerebral infarctions (Table S1 in the supplemental information online). As described in AD, this may be mainly related to impairment in BLB production in the brain, rather than reduced supply from the blood to the brain, as has been described for other neurological diseases (see below).

Oxidative imbalance is a common feature of neurological conditions due to the high lipid content and oxygen consumption, and limited antioxidant mechanisms in the brain. In brain trauma [59], hemorrhage and hypoxic/ischemic conditions [60], heme released into extracellular spaces may contribute to vasospasms and induce oxidative stress leading to cell death [59–61] (Figure 3). Inside the cell, heme becomes a substrate for the constitutive HMOX2, which is highly and widely expressed in neurons (Figure 3). The protection may thereafter be potentiated by HMOX1 induction [59,60], by heme itself, or by the carbon monoxide (CO)-mediated Nrf2 interaction with antioxidant response elements on the *HMOX1* gene promoter [62]. As a result, *HMOX1* expression is upregulated in astrocytes, macrophages, endothelial cells [63], and in microglia surrounding brain lesions [61]. HMOX1 modulation contributes to limiting lipid peroxidation, ameliorating brain damage and improving recovery from cell loss and motor impairment [59].

Several *in vivo* observations suggest a differential role for HMOX2 and HMOX1 in the central nervous system (CNS). As shown in a knockout mouse model, HMOX2 is protective (see above),

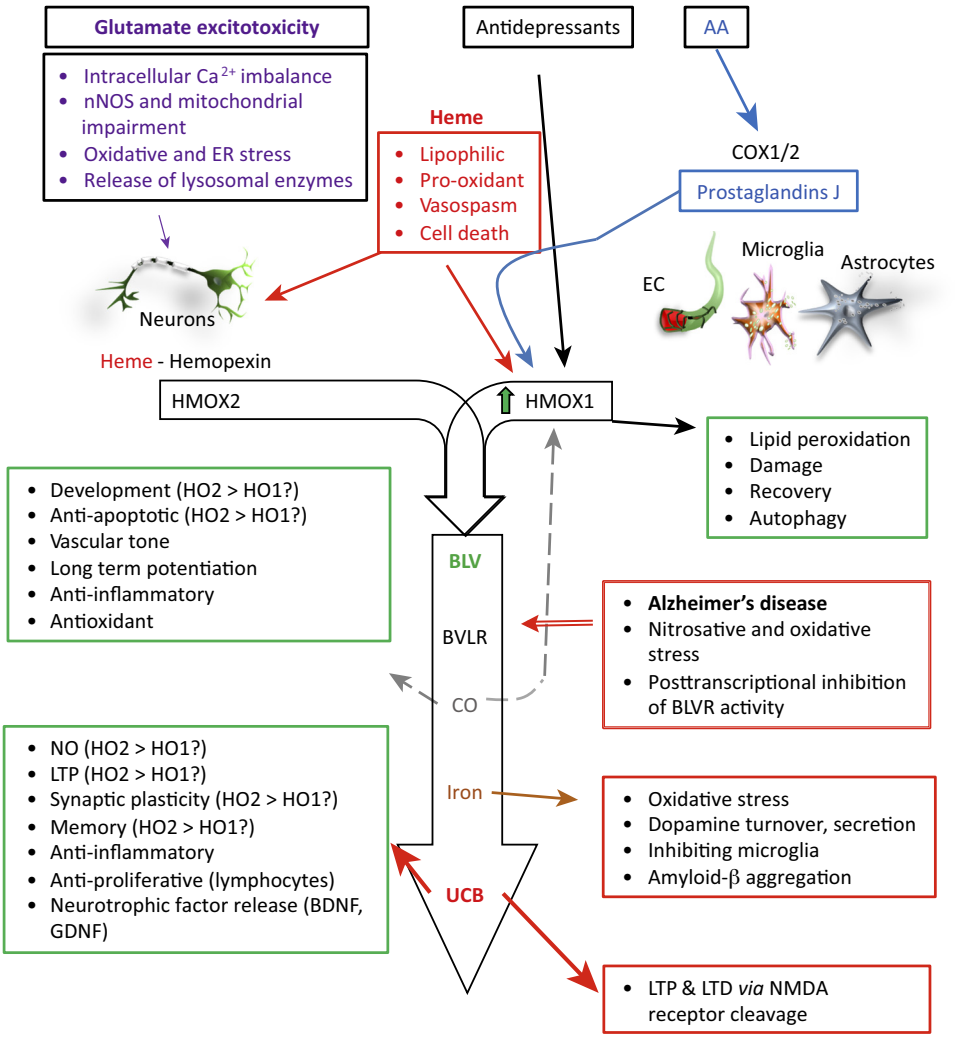

Figure 3. Molecular and Functional Impact of the Yellow Players on the Central Nervous System. The schematic diagram illustrates identified pathways involved in heme-induced regulation of heme oxygenase 1 (HMOX) activity and its subsequent effects. The effects are depicted of antidepressants, arachidonic acid (AA), and glutamate excitotoxicity on HMOX1 pathways in cells of the central nervous system. The downstream byproducts of these pathways, biliverdin (BLV), iron, carbon monoxide (CO), and unconjugated bilirubin (UCB), can exert various biological effects (molecular and pathological consequences); the green boxes are suggestive of positive biological effects and the red boxes, negative biological effects from each of the yellow players. Abbreviations: BDNF, bone-derived neurotrophic factor; BLVR, biliverdin reductase; COX1/2, cyclooxygenase; EC, endothelial cells; ER, endoplasmic reticulum; GDNF, glial cell-derived neurotrophic factor; HO1/2, heme oxygenase; LDP, long-term depression; LTP, long-term potentiation; nNOS, neuronal nitric oxide synthase.

whereas the induction of HMOX1 may be harmful, and its role different in other organs [63]. HMOX2 may participate via CO in the development and inhibition of apoptosis in both primary cell cultures and *in vivo* ischemic and traumatic brain injury [64]. By increasing NO levels, CO also impacts the long-term potentiation of signal transmission in the hippocampus, vessel tone, anti-inflammatory, and antioxidant activity, and modulates the activity of soluble guanylate cyclase, opening calcium-activated potassium channels [65]. These results were demonstrated by exposing rodents or cells to CO, or by modulating HMOX activity *in vitro* [65].

Heme oxygenase activity counteracts glutamate excitotoxicity [66], another major mechanism of neuronal damage (Figure 3), both *in vitro* and *in vivo* in murine *HMOX1*-knockout models [67].

The protective activity of HMOX2 is due to BLB and inducible NO synthase (iNOS) expression and NO production acting on synaptic plasticity, improving memory processes (reviewed in [64]) and specifically reducing apoptosis but not necrosis [68]. These effects are present at low BLB concentrations [25–50 nanomolar free bilirubin (Bf)] in primary neuronal and granular cells [68]. Comparable BLB concentrations (3–30 nanomolar Bf for 24–48 h) impaired **long-term potentiation** (LTP) and **long-term depression** (LTD) in rat hippocampal organotypic cultures by calpain-mediated proteolytic cleavage of NMDA receptor subunits NR1, NR2a, NR2b, without altering interleukin (IL)-1β, or tumor necrosis factor (TNF)-α secretion [69]. Thus, the loss of neurons in the CNS results in the loss of constitutive HMOX2, which further increases cellular damage.

BLB has also potent anti-inflammatory activities in brain tissue. This effect has been well documented in experimental autoimmune encephalomyelitis (EAE), a rodent model of multiple sclerosis [4]. *in vitro*, 20–150 μmol total BLB inhibited T cell proliferation, and IL2, TNFα, IL4, and IL10 release, as well as MHC class II expression in macrophages via NF-κB signaling. The beneficial effect of BLB was confirmed *in vivo* by the reduction of the above-mentioned markers of inflammation and neurological damage when the serum BLB level was increased by eight- to tenfold in EAE rats [4]. Consistent with anti-inflammatory activity, increased release of brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) has been reported to lead to reduced neuronal loss in the substantia nigra in animal models of Parkinson disease (PD) via extracellular signal-regulated kinases (ERK), phosphatidylinositol-4,5-bisphosphate 3-kinase–protein kinase B (PI3K-Akt), and NF-κB signaling [70]. In turn, **J series prostaglandins (PGs)** have been shown to induce HMOX1 and protect primary mouse neuron cultures from oxidative stress [71]. HMOX1 induction has also been found to increase autophagy *in vitro*, a controlled modality of cell death [72].

From another perspective, brain deposition of iron, an important pathogenic factor in many diseases, has been documented for AD lesions, and shown to accelerate amyloid-β (Aβ) aggregation and increased cell death *in vitro* in cell lines exposed to Aβ fibrils in the presence of varying iron concentrations [73]. In AD, the upregulation of HMOX1/BLVRA axis represents the early response to increased oxidative, nitrosative, and inflammatory reactions documented in the brains of patients with AD [64]. Nevertheless, compared with other organs, the brain has a limited capability to counteract oxidative stress, and oxidative and nitrosative stress may lead to structural modifications in cellular enzymes. BLVRA appears to be especially sensitive to this effect, being functionally inactivated via reduced phosphorylation and autophosphorylation (necessary for its functions) despite the upregulation of its mRNA and protein levels. Consequently, the regeneration of cytoprotective BLB by the HMOX/BLVR cycle may be disrupted, resulting in damage and disease progression [64]. Indeed, high concentrations of iron in the rat brain have been observed to rapidly increase NF-κB DNA binding, which may contribute to limiting oxygen reactive species-dependent damage by impacting the activity of the antioxidant enzyme, catalase [74].

Concluding Remarks

Here, we present several lines of evidence to support the notion that BLB and all the machinery involved in its production and metabolism (the yellow players) are deeply involved in several crucial steps of cellular pathways and homeostasis. As shown in Figure 2, this occurs by a complex, intricate network involving several genes and pathways, indicating that BLB and related enzymes have more important functions than merely representing waste products, as had been described during the 1980s. They may undoubtedly represent fundamental players in

Outstanding Questions

What is the protective threshold level of UCB in different cells and/or tissues? Could various pathologies respond differently to similar UCB concentrations? Could different UCB levels be necessary to reach similar protection statuses in various tissues and/or diseases?

BLVR activity appears to be especially affected by nitrosative/oxidative post-translational modifications, which impair its activity. Could shielding BLVR from nitrosative/oxidative effects preserve its protective capability? Could the pharmacological induction of HMOX1 uniquely target and increase BLV/UCB concentrations to trigger protection? Would it be more important to equilibrate HMOX and BLVR activities? Would it be fruitful to begin evaluating specific BLVR inducer(s)?

Could the dissection of the direct BLV/UCB and indirect signaling pathways involving the yellow players and their biological roles provide new putative therapeutic targets to treat a variety of diseases and conditions?

HMOX2 is highly expressed (specifically) in neurons, which are lost in various neurological diseases. Could HMOX2 be implicated in disease progression? Should it be reconsidered in the pathogenesis of neurological diseases?

Could the anti-inflammatory and antioxidant properties of the yellow players also lead to 'adverse effects' under specific circumstances?

both health and disease, although future experiments are required to validate many of their putative functions, particularly in humans (see Outstanding Questions). Interestingly, the antioxidant, anti-inflammatory, antiproliferative, and immunomodulatory activities of BLB and the yellow players lead to the intriguing idea that the regulation of, and by, these molecules could be used in preventative or therapeutic modalities in several common conditions, including metabolic, cardiovascular, oncogenic, and neurological disorders, as discussed here.

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