“Iatrogenic Gilbert syndrome”— A strategy for reducing vascular and cancer risk by increasing plasma unconjugated bilirubin

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Summary The catabolism of heme, generating biliverdin, carbon monoxide, and free iron, is mediated by heme oxygenase (HO). One form of this of this enzyme, heme oxygenase-1, is inducible by numerous agents which promote oxidative stress, and is now known to provide important antioxidant protection, as demonstrated in many rodent models of free radical-mediated pathogenesis, and suggested by epidemiology observing favorable health outcomes in individuals carrying high-expression alleles of the HO-1 gene. The antioxidant impact of HO-1 appears to be mediated by bilirubin, generated rapidly from biliverdin by ubiquitously expressed biliverdin reductase. Bilirubin efficiently scavenges a wide range of physiological oxidants by electron donation. In the process, it is often reconverted to biliverdin, but biliverdin reductase quickly regenerates bilirubin, thereby greatly boosting its antioxidant potential. There is also suggestive evidence that bilirubin inhibits the activity or activation of NADPH oxidase. Increased serum bilirubin is associated with reduced risk for atherogenic disease in epidemiological studies, and more limited data show an inverse correlation between serum bilirubin and cancer risk. Gilbert syndrome, a genetic variant characterized by moderate hyperbilirubinemia attributable to reduced hepatic expression of the UDP-glucuronosyltransferase which conjugates bilirubin, has been associated with a greatly reduced risk for ischemic heart disease and hypertension in a recent study. Feasible strategies for boosting serum bilirubin levels may include administration of HO-1 inducers, supplementation with bilirubin or biliverdin, and administration of drugs which decrease the efficiency of hepatic bilirubin conjugation. The well-tolerated uricosuric drug probenecid achieves non-competitive inhibition of hepatic glucuronidation reactions by inhibiting the transport of UDP-glucuronic acid into endoplasmic reticulum; probenecid therapy is included in the differential diagnosis of hyperbilirubinemia, and presumably could be used to induce an “iatrogenic Gilbert syndrome”. Other drugs, such as rifampin, can raise serum bilirubin through competitive inhibition of hepatocyte bilirubin uptake — although unfortunately rifampin is not as safe as probenecid. Measures which can safely achieve moderate serum elevations of bilirubin may prove to have value in the prevention and/or treatment of a wide range of disorders in which oxidants play a prominent pathogenic role, including many vascular diseases, cancer, and inflammatory syndromes. Phycobilins, algal biliverdin metabolites that are good substrates for biliverdin reductase, may prove to have clinical antioxidant potential comparable to that of bilirubin.

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Antioxidant role of heme oxygenase

Heme oxygenase has emerged in recent research as an antioxidant enzyme of great physiological importance [1,2]. There are at least three isoforms of this enzyme in mammals; two of these are constitutively expressed, but heme oxygenase-1, the most important from the standpoint of antioxidant protection, is inducible. Heme oxygenase functions to cleave heme, converting it to carbon monoxide, free ferrous iron, and the linear tetrapyrrole biliverdin; the latter is rapidly reduced to bilirubin by the ubiquitously expressed biliverdin reductase. Heme it is synthesized in all nucleated cells, reflecting the fact that this crucial cofactor is not exchanged between tissues. Heme oxygenase activity promotes recycling of tissue iron; when tissue heme levels increase, heme interacts with the inhibitory transcription factor Bach1, disinhibiting HO-1 transcription and thus promoting heme catabolism [3,4]. This effect is also protective, since elevated heme levels can induce oxidant stress. On the other hand, since heme plays a central role in aerobic energy metabolism, hypoxia promotes increased production of Bach1, suppressing HO-1 transcription and thereby conserving heme. The antioxidant function of HO-1 is primarily attributable to the fact that bilirubin is a potent and highly versatile antioxidant, scavenging superoxide, peroxyl radicals, hydroxyl radicals, hypochlorous acid, and singlet oxygen, as well as the reactive nitrogen species nitroxyl and peroxynitrite; [5–10] furthermore, bilirubin can reduce the α-tocopheroxyl radical, thereby promoting recycling of vitamin E [10]. This presumably explains why the HO-1 promoter has evolved to respond to oxidant stress via antioxidant response elements; the transcription factor Nrf2, whose half-life and nuclear access is increased by oxidants and electrophiles that covalently modify cysteine residues in its binding protein Keap1 [11,12], competes with Bach1 for heterodimerization with Maf transcription factors, producing heterodimers which boost HO-1 transcription by binding to multiple antioxidant response elements (also known as ’’Maf recognition elements’’) in the HO-1 promoter [3]. Phase II inducer chemicals, as well as a wide variety of agents which promote or mediate oxidant stress – cytokines, endotoxin, hyperoxia, ultraviolet light, X-irradiation, hydrogen peroxide, peroxynitrite, taurine chloroamine, phorbol esters – have been shown to act as HO-1 inducers [13–15]. Other agents reported to induce HO-1 in various types of cells include cGMP, statins, alanine, pro- bucol, propofol, and acetylcarnitine [16–22]; interferon-γ and desferrioxamine, like hypoxia, induce Bach1 and thus inhibit HO-1 expression [3,4].

The carbon monoxide produced by HO-1 activity is also suspected to contribute to the protection afforded by this enzyme. CO can suppress activation of NF-kappaB, thereby exerting anti-inflammatory effects [23]. Moreover, like nitric oxide (NO), it can activate the soluble guanylate cyclase [24]; however, since it increases the Vmax of this enzyme far less than does NO, its ability to compete with NO for binding to the active site heme implies that CO can actually decrease guanylate cyclase activity when NO is amply available [25]. This presumably explains why selective overexpression of HO-1 in vascular smooth muscle cells of mice was associated with increased blood pressure and decreased arterial responsiveness to NO [26]; likewise, increased arterial expression of HO-1 in Dahl salt-sensitive rats is a mediator of their hypertension [27]. On the other hand, HO-1-derived CO can promote vasodilation in the context of low NO availability [28]. While there is general agreement that CO may collaborate with bilirubin in achieving anti-inflammatory effects, in many studies bilirubin has been shown to be the chief mediator of HO-1’s protective actions.

When cells or tissues are exposed to oxidant stressors, concurrent administration of an HO-1 inhibitor typically exacerbates the resulting cellular trauma [29–33]; this clearly indicates that HO-1 induction represents an important physiological mechanism for coping with oxidant stress. Moreover, HO-1 knockout mice, as well as cells derived from such mice, are more sensitive to oxidant stress. When HO-1 is inhibited in cell culture, addition of bilirubin in nanomolar concentrations has often been found to restore the protection that otherwise would be mediated by HO-1 activity; this suggests that bilirubin may be the key mediator of HO-1’s antioxidant benefits.

Snyder and colleagues have noted that, since tissue levels of free bilirubin are usually in the low nanomolar range, it is difficult to understand how such low levels of bilirubin could scavenge much higher concentrations of oxidants. They have attempted to resolve this paradox by proposing that oxidants preferentially extract electrons from the C-10 methylene group of bilirubin, reconvert it to the lower energy, resonance stabilized compound biliverdin; the latter is then rapidly reduced back to bilirubin by biliverdin reductase. Thus, they propose that, much like glutathione, bilirubin can be reutilized indefinitely as an oxidant quencher thanks to its enzymatic reductive mechanism [34,35]. Consistent with this view, this group has shown that inhibition of biliverdin reductase with...
small interfering RNA markedly boosts oxidant levels in cells — indeed, to a greater degree than when glutathione synthesis is blocked. They also report that as little as 10 nM of albumin-bound bilirubin can protect cells from 100 μM hydrogen peroxide — an effect which presumably demonstrates the rapid turnover of the bilirubin–biliverdin cycle. This hypothesis provides a satisfying rationale for the otherwise rather inexplicable fact that mammals go to the trouble and energetic expense of converting biliverdin — a soluble, readily excreted compound with antioxidant activity of its own — to poorly soluble and potentially toxic bilirubin. Nonetheless, Stocker emphasizes that not all reactions between bilirubin and oxidants generate biliverdin [10] — as in the case of peroxy radicals, for example [5]. On the other hand, Kaur et al., report production of biliverdin when bilirubin reacts with active nitrogen species, and comment approvingly on the Snyder thesis [9].

**Protective potential of HO-1 confirmed**

The likelihood that HO-1 activity has an important impact on the pathogenesis of various diseases finds strong support in rodent studies as well as in epidemiology. Increasing tissue activity of HO-1, either by transgenic methods or by treatment with HO-1 inducers, has protected rodents from atherogenesis, thrombosis, renal, hepatic, or pulmonary injury, ischemia—reperfusion injury, and angiotensin II-mediated cardiac hypertrophy; in many of these studies, concurrent administration of HO-1 inhibitors abrogated this protection [36–54]. Conversely, HO-1 knockout mice are more prone to atherogenesis and vein graft stenosis and calcification [55]. HO-1 activity appears to be of particular importance in endothelial cells; increased endothelial HO-1 activity suppresses induction of adhesion factors and monocyte transmigration by cytokines, oxidants, and oxidized LDL, prevents the sloughing of endothelial cells in diabetic rats, and diminishes angiotensin II-mediated DNA damage [29,31,33,56–59]. Indeed, it may not be exaggerating to state that, in rodents, up-regulation of HO-1 activity has been found to confer protection in virtually every type of free radical-mediated pathology in which it has been studied — a tribute to the potency and versatility of the antioxidant activity of bilirubin (albeit CO has contributed to the observed protection in some instances).

The impact of HO-1 expression on human pathologies can be readily assessed through epidemiology, thanks to the fact that a common polymorphism in the promoter region of the HO-1 gene has a notable impact on gene expression. In humans, this promoter contains a GT dinucleotide repeat of variable length (GT)n. The most commonly encountered lengths are of 23 and 30 repeats. The expression of genes containing 25 or fewer repeats has been found to be greater than that of genes containing over 25 repeats [60–62], presumably because DNA with a greater number of GT repeats can twist into a ‘‘Z’’ configuration that is unfavorable for transcription [63]. Epidemiologists have thus stratified subjects into those with a small (S) repeat promoter — associated with increased expression of HO-1 — and those with a large (L) repeat promoter, and have then assessed the frequency of these genes types in subjects afflicted with various pathologies. Perhaps not surprisingly, the L type gene has been found to be relatively more common in cases than controls in studies examining risk for emphysema (in smokers), coronary artery disease, abdominal aortic aneurysms, restenosis after coronary stenting or peripheral angioplasty, kidney allograft failure, oral squamous cell carcinoma in betel nut chewers, and lung adenocarcinoma in male smokers [3,64,65]. To date, no such relationship has been seen with respect to myocardial infarction, Alzheimers, or Parkinson disease [60,66]. Research of this type is still in an early stage, so many more relevant findings can be expected in the near future.

Perhaps the most intriguing such study focused on longevity in the Japanese population. Sasaki and colleagues determined the frequency of S, M (mid-size repeats) and L alleles as a function of age in 512 healthy subjects [67] in males, the frequency of the class L allele (defined as genotype L/L, L/M, or L/S) declined significantly and markedly as a function of age (<60y, 23%; 60–74y, 16%; >75y, 10%). In women, the decline did not achieve significance (<60y, 22%; 60–74y, 22%; >75y, 15%). (Arguably, the trend in the women might have achieved significance if a higher age bracket had been included in the analysis; the average lifespan of Japanese women is now 86 years, and little enrichment of the higher expression alleles could be expected until an ample fraction of the women had died.) These findings thus suggest that up-regulation of HO-1 activity may provide protection from all-cause mortality.

**Prognostic significance of serum bilirubin**

A related strain of epidemiological research has attempted to correlate serum bilirubin levels with...
endpoints related to atherosclerosis — coronary or carotid stenosis, ischemic heart disease, and cardiovascular death. The first such study, by Schwertner and colleagues, examined the prevalence of subclinical coronary stenosis (≥50%) as a function of bilirubin level; a marked inverse association was noted. Relative risk in the four quartiles of serum bilirubin was found to be 1.0, 0.6, 0.4, and 0.3 [68]. The striking findings of this study encouraged a number of other groups to undertake analogous investigations. A sufficient number of such studies have become available to enable a recent meta-analysis by Novotny and Vitek, incorporating 11 suitable studies, of both cross-sectional and prospective design [69]. The authors conclude that "a close negative relationship was found between serum bilirubin levels and severity of atherosclerosis (Spearman rank coefficient r = −0.31, P = 0.0001). Unambiguous inverse relationship between serum bilirubin levels and atherosclerosis was demonstrated in this preliminary meta-analytic study." In dealing with a study that had demonstrated a U-shaped relationship between serum bilirubin and ischemic heart disease incidence (i.e. higher risk with high bilirubin) [70], the authors showed that the data from this study fit the overall pattern if patients experiencing liver failure (elevated liver enzymes) were excluded from the analysis.

In light of the fact that oxidants are prominent mediators of "spontaneous" mutagenesis, a few studies have examined the relationship of HO-1 polymorphisms or of serum bilirubin to cancer risk. In a prospective Belgian study, comparing the highest (≥6mg/L) vs. the lowest (<2mg/L) categories of serum bilirubin, relative risk of cancer mortality was 0.42 for men (significant) and 0.76 for women (non-significant) [71]. (Surprisingly, bilirubin did not predict cardiovascular mortality in this study, whose findings were incorporated into the meta-analysis cited above.) In a study utilizing data from the Third National Health and Nutrition Examination Survey, an increase of serum bilirubin of 1 mg per dl was associated with a relative risk of prevalent non-dermatological cancer of 0.81 and of prevalent colon cancer of 0.257 (95% CI 0.254–0.260); the authors postulated that enterohepatic circulation of bilirubin, exposing colonic mucosa to relatively high levels, accounted for the unusually strong seeming impact on colon cancer risk [72]. On the other hand, a previous report saw no such association between bilirubin and colon cancer risk [73]. In a case–control study of breast cancer, risk associated with serum bilirubin in the upper quartile, relative to the lower quartile, was 0.50 (95% CI 0.26, 0.97) [74]. Finally, as noted above, the high expression form of HO-1 has been associated with reduced risk for squamous oral cancer in betel nut chewers [75]. Thus, although relevant data are still somewhat sparse, the few studies that exist are encouraging in regard to the possible impact of bilirubin on cancer risk.

With respect to studies correlating increased serum bilirubin with better health outcomes, it is important to ask: what is the significance of elevated bilirubin? Is it merely serving as a marker for increased HO-1 activity in tissues, associated with an increased whole-body production rate of heme and bilirubin? Or do these correlations also reflect the fact that increased serum bilirubin — possibly stemming from decreased efficiency of hepatic conjugation and excretion of this antioxidant, or from increased erythrocyte turnover — can boost tissue levels of bilirubin sufficiently to provide important antioxidant protection? While the latter may seem intuitively obvious, it should be noted that serum bilirubin, because of its poor solubility, is almost wholly bound to albumin; only a very tiny fraction of unconjugated plasma bilirubin is in free form. Indeed, it previously was thought that only 0.005% of total plasma unconjugated bilirubin was unbound [76]; this would imply a free plasma bilirubin level so low that its ability to influence tissue bilirubin levels meaningfully would be doubtful. However, earlier studies did not account for the fact that affinity of albumin for bilirubin declines substantially as albumin concentration increases. More recent work suggests that, at a physiological adult albumin concentration of 600 µM, the affinity constant for bilirubin is approximately 2.3 × 10^6 L mol⁻¹ [77,78]; this implies that, at a typical adult serum unconjugated bilirubin concentration of 10 µM, the concentration of unbound bilirubin will be about 7.4 nM, a level which is meaningful relative to the tissue level of 10–50 nM (much of it presumably associated with membranes or hydrophobic sites on proteins) reported by Snyder et al [35]. In the diacid form which predominates at physiological pH, free bilirubin readily passes through membranes to enter or exit cells [78,79]. And there can be no doubt that, at pathological plasma levels (>200 µM), plasma bilirubin can raise tissue levels sufficiently to give rise to kernicterus in newborns.

An alternative or adjunctive possibility is that decreased serum bilirubin may be a marker for a metabolic state that promotes vascular disease. Several cross-sectional studies have found that serum bilirubin correlates inversely with risk factors associated with insulin resistance syndrome, including serum insulin, triglycerides, systolic blood pressure, apolipoprotein B, and adiposity [80–82].
Do elevated insulin levels influence bilirubin metabolism, or, conversely, do elevated bilirubin levels promote insulin sensitivity and discourag weight gain? In this regard, insulin can decrease expression of delta-aminolevulinic acid synthase — the rate-limiting enzyme for heme production — in hepatocytes [83]; whether it does so in other tissues (most heme is synthesized in bone marrow erythroblasts) is not known. Even if insulin resistance syndrome does somehow suppress serum bilirubin, this is unlikely to provide the entire explanation for the association of low bilirubin with elevated coronary risk, as many studies found that this association persisted after statistical adjustments for known cardiovascular risk factors. A similar comment could be made with respect to evidence that smokers tend to have decreased bilirubin levels [80,81].

Data pertaining to Gilbert syndrome strongly suggest that moderately increased plasma free bilirubin can indeed provide important antioxidant protection for tissues. Gilbert syndrome is an innocuous recessive genetic variant in which hepatocytes express decreased amounts (~30% of normal) of UDP-glucuronosyltransferase type 1A1 (UGT1A1), the enzyme almost solely responsible for bilirubin conjugation; the mutation is in the promoter region, leading to decreased expression of an enzyme with normal structure and specific activity [84]. As a result, serum unconjugated bilirubin tends to be elevated by 2–3-fold; serum bilirubin is typically about 30 μM in Gilbert subjects. (No known adverse effects are associated with Gilbert syndrome, notably distinguishing it from type 1 Crigler–Najjar syndrome, in which UGT1A1 activity is absent owing to a mutant enzyme, and serum bilirubin is an order of magnitude higher, giving rise to kernicterus and other severe complications.) [85] Czech researchers recently recruited 50 subjects with Gilbert syndrome, of age 40 and older; average age of the group was 50; they observed that only one member of this group — 2% (0.05–10.7%, 95% CI) — had symptomatic ischemic heart disease [86] In contrast, prevalence of coronary disease in an age- and sex-matched control group was found to be 12%, and the authors state that prevalence of coronary disease in this age group among Czech subjects, as described in the literature, tends to be 10–20%. Moreover, this one case of coronary disease appeared to be atypical, in that neither the coronary nor carotid arteries were detectibly stenotic. Perhaps more astonishing was the fact that only 1 of the 50 Gilbert subjects was hypertensive. While it would be rash to read too much into one small study — attempts to confirm this finding in other populations should be an urgent priority — these results, if confirmable, imply that maintaining optimal serum bilirubin levels may have remarkable potential as a strategy for preserving vascular health. Viewed in the context of the very versatile and potent protection afforded by up-regulation of HO-1 activity in rodent studies, this conclusion is at least reasonably credible.

The Gunn rat is characterized by hyperbilirubinemia attributable to a complete absence of UGT1A1 activity; it may thus be viewed as a model of the most severe type of Crigler–Najjar syndrome. A recent study shows that Gunn rats are substantially protected from the adverse impact of angiotensin II infusion on blood pressure and endothelial function [87]. In particular, the ability of angiotensin II to raise the endothelial dihydriobioterin/tetrahydrobioterin ratio — thereby compromising NO synthase activity — is virtually abolished.

Why is bilirubin such an important antioxidant?

Albumin-bound bilirubin makes a substantial contribution to plasma antioxidant activity [88]. Bilirubin is bound in such a way that the C-10 methylene group can readily donate electrons to plasma oxidants [10]; thus, it may help to protect LDL particles from plasma oxidants.

But how is it that intracellular bilirubin — in a concentration not exceeding 50 nM - can have an important impact on free radical damage? It is not likely that, in such low concentrations, bilirubin may be especially adept at ‘‘fixing’’ the primary oxidant damage induced by these oxidants in proteins, lipids, and DNA [90]. Many of these lesions may have a relatively long half-life, and their evolution into irreparable lesions may require additional chemical events — such as a reaction with another molecule, or intramolecular rearrangements. Thus, there may be time for bilirubin to encounter these primary lesions before they lead to permanent damage. Furthermore, these primary lesions may be relatively weak oxidants — compared to hydroxyl radical or nitrogen dioxide, for example — so they would be more likely to preferentially extract electrons from bilirubin’s C-10 methylene, an eager electron donor. Thus, biliverdin would be the chief product of bilirubin’s antioxidant activity, enabling bilirubin to be recycled.
But these considerations are still dodging an important issue — how can intracellular free bilirubin, in concentrations in the low nanomolar range, compete effectively as a radical scavenger with other intracellular antioxidants — including ascorbate, urate, and reduced sulfhydryls — that are present in millimolar or near-millimolar concentrations? The efficacy of a scavenger is contingent on the rate at which it can make contact with oxidants, and that rate can be expected to be proportional to the concentration of the scavenger. The intracellular concentration of bilirubin, even after HO-1 induction, can be expected to be over 10,000 times lower than that of ascorbate, another versatile radical scavenger. The fact that, as Snyder has emphasized, reversible reduction by biliverdin reductase multiplies the scavenging potential of the bilirubin pool, does not alter the fact that the size of this pool intracellularly is quite small compared to that of other effective physiological scavengers. What makes bilirubin such a remarkably effective antioxidant?

The likely explanation is that bilirubin’s chief role is not to act as a radical scavenger, but rather as a potent and specific inhibitor of the membrane-bound NADPH oxidase, a key source of oxidants not only in phagocytes, but in a high proportion of non-phagocytic cells as well [91]. Indeed, the results of a number of studies can be interpreted as evidence that HO-1 induction and/or bilirubin suppress superoxide production under circumstances in which the prime source of superoxide is likely to be NADPH oxidase [92–99,54,100,101]; direct scavenging of superoxide by bilirubin is not a likely explanation for these findings, since bilirubin’s activity in this regard is unremarkable — less than that of ascorbate [6,8,10], which is present in much higher concentrations. Nonetheless, the authors of these studies have usually attributed bilirubin’s potent antioxidant activity to its radical scavenging activity. It is therefore gratifying to note that, in a recent study, Boczkowski and colleagues, having demonstrated that bilirubin can inhibit the membrane translocation of p47, an essential step in activation of this enzyme complex.

This new understanding of bilirubin’s efficacy can yield an elegantly simple explanation for the antioxidant physiological role of HO-1: Excessive intracellular oxidant activity triggers induction of HO-1; the resulting intracellular generation of bilirubin (via biliverdin and biliverdin reductase activity) provides feedback suppression of NADPH oxidase activity, one of the cell’s chief sources of oxidants. Moreover, the concurrently generated CO may contribute at least modestly to this inhibition of NADPH oxidase [106]. And this CO can “pinch-hit” for the nitric oxide scavenged by interaction with excess superoxide; like NO, CO is an activator of guanylate cyclase. Finally, diminution of the heme pool, in the longer term, may suppress NADPH oxidase activity by decreasing availability of its heme-dependent gp91phox subunit [97]. Thus, induction of HO-1 can work in multiple complementary ways to provide feedback compensation for the consequences of excessive NADPH activation. The role of biliverdin reductase is to generate bilirubin and to maintain it in its low-solubility conformation in the face of oxidant assault.

Since NADPH oxidase plays a prominent role in the anti-infective activity of white cells, it is reassuring to note that clinicians have not reported any evident increase in risk for infection in Gilbert subjects; thus, moderate down-regulation of NADPH oxidase activity may be compatible with adequate immune protection. Nonetheless, Haga and colleagues, having demonstrated that bilirubin can suppress lymphocyte-mediated cytotoxicity, refer to "the increased susceptibility to infection observed in hyperbilirubinemic patients" [107,108]. Other relevant observations are noted below.
Strategies for harnessing Bilirubin's health protective potential

How can the versatile antioxidant benefits of bilirubin be exploited in prevention and therapy? One evident approach is to administer agents which have HO-1 inductive activity in target tissues. Phase II inducers, such as sulforaphane, lipoic acid, green tea polyphenols, and various other bioavailable flavonoids have evident potential in this regard. The intriguing report that alanine has HO-1 inductive activity [19] should be followed up, and the extent to which the poorly understood vascular-protective activity of probucol [109–114] may be mediated by HO-1 induction [20] should be explored.

Biliverdin and phycobilins as antioxidant nutraceuticals

Since bilirubin undergoes enterohepatic circulation (free but not conjugated bilirubin is readily reabsorbed [115]), oral administration of bilirubin — or, preferably, of its precursor biliverdin, which is much more soluble and likely would be converted to bilirubin in the intestinal mucosa - has the potential to raise serum and tissue bilirubin levels. A handful of studies have in fact evaluated the impact of biliverdin supplementation in rodents. Back in 1993, Nakagami and colleagues reported that oral administration of biliverdin (5 mg/kg) an hour before injection of Forssman antiserum suppressed the resulting anaphylaxis and cut the death rate in half; this effect was thought to stem from biliverdin's ability to inhibit complement activation [116]. Much more recently, Bach and colleagues have reported that biliverdin, administered orally at a dose of 50 μmol/kg 2–3 times daily, greatly increased cardiac allograft survival in mice; two-thirds of the mice accepted the grafts and developed long-term tolerance to the transplanted alloantigens, whereas all grafts in the control group were rejected in a mean time of 11.5 days [117]. This effect was believed to reflect down-regulation of NF-kappaB activation in T lymphocytes. Hopefully, studies will soon be forthcoming evaluating the impact of oral biliverdin on vascular function.

Endogenous production of heme has been estimated at 300–400 mg daily [118] — giving rise to a nearly equivalent amount of bilirubin. Only a portion of an orally administered biliverdin dose would be absorbed, and first-pass hepatic metabolism would clear some of this. Thus, it can be anticipated that supplemental daily intakes of 500 mg or more might be required to make a clinically meaningful impact on serum and tissue bilirubin levels. The current commercial source of bilirubin, ox bile, would presumably be inadequate if millions of people wanted to use effective doses of biliverdin at an affordable cost. Chemical synthesis of biliverdin is presumably complex and costly. Thus, developments in biotechnology or organic chemistry will be required before biliverdin supplementation could achieve its optimal health promoting potential.

An alternative strategy is suggested by the fact that plants, algae, and cyanobacteria manufacture compounds known as phycobilins that are close structural analogs of biliverdin — reflecting the fact that they are biliverdin metabolites. [119] Phycobilins are ligated to apoproteins to generate protein-chromophore complexes known as phycocyanins, which function to "harvest" visible light in chloroplasts. The three most prominent phycobilins are phycocyanobilin (blue), phycocyanobilin (red), and phycoerythrobilin (red); they are synthesized from biliverdin by reduction reactions and isomerizations which produce a range of structures that absorb different portions of the visible spectrum, and that are capable of being enzymatically ligated to apoproteins. However, these structural alterations are fairly trivial, affecting only the ends of the molecules, such that the central C-10 region remains unaltered and the substantial conjugation of the electronic structure of biliverdin is largely preserved. Thus, phycobilins and phycocyanins have shown antioxidant activity in vitro, analogous to that of biliverdin [120–126]. Moreover, Terry and colleagues have demonstrated that phycobilins are good substrates for biliverdin reductase, with $K_m$'s similar to those of biliverdin, and $V_{max}$s about half as high [127].

Reduction of phycobilins gives rise to a set of compounds ("phycorubins") that are structural analogs of bilirubin, and that seem likely to possess somewhat comparable physiological activity. Indeed, this may help to explain why oral administration of algae or of phycocyanin has shown remarkably versatile and potent antioxidant, anti-inflammatory, and anti-allergic activities in a number of rodent studies [128,129,130,131]. In particular, it is noteworthy that the impacts of parenteral bilirubin and of oral phycocyanin on endotoxin shock in rats are quite parallel — survival is enhanced owing to a suppression of iNOS induction; [102,132] inhibition of NAPDH oxidase activation is the likely explanation for this phenomenon. Hence, by harnessing the biosynthetic capacity of algae, it may prove feasible to develop phycobilins as nutraceutical antioxidants capable of replicating the range of health benefits conferred by
elevated bilirubin levels. Indeed, Inoguchi has recently observed that phycocyanobilin dose-dependently inhibits the NADPH oxidase activity of human cell cultures (endothelial, smooth muscle, and mesangial) in the concentration range 1–20 μM; the inhibitory effects of biliverdin are quite similar in these cell cultures (Toyoshi Inoguchi, personal communication).

Phycocyanins constitute a high proportion of the total protein content of many algae; [126] moreover, when algae grown in the dark are fed delta-aminolevulinic acid, they produce and secrete free phycobilins [133]. Phycobilins can be extracted from algae and cyanobacteria by prolonged treatment with boiling methanol, which cleaves the thioether linkage between phycobilins and their apoproteins; a relatively selective extraction can be achieved if algae are pre-extracted with cold methanol, which will remove other chromophores such as chlorophyll and carotenoids [134–136].

Spirulina is one of the richest known natural sources of phycocyanobilin, and is used commercially for production of phycocyanin (employed as a food dye and as a chromophore tag in biological research) [137,138]. Unfortunately, since Spirulina is obligately phototrophic, it is difficult to produce efficiently in bulk; organisms that can be grown heterotrophically might ultimately have greater potential for commercial phycocyanin production [139,140].

An additional possibility is that genetic engineering could be employed to convert chlorophyll-rich plants into rich sources of biliverdin and phycocyanobilin. Although most of the protoporphyrin IX produced in green plants is used for chlorophyll synthesis, a small portion of it is converted to phytochromobilin. Phytochromobilin is the chromophore for phytochrome proteins which act as light detectors that regulate plant development (rather than harvesting light for biosynthesis, as in algae) [141]. Insertion of Mg(2+) into protoporphyrin IX, catalyzed by Mg-chelatase, commits it to chlorophyll synthesis, whereas insertion of Fe(2+) via the enzyme ferrochelatase generates heme [142]. Mg-chelatase is at a marked competitive advantage in this regard, in as much as it is Km for protoporphyrin – 13 nM – is orders of magnitude lower than that of plant ferrochelatase, determined to be 2.4 μM in peas [143]. In contrast, the ferrochelatase of non-photosynthetic organisms appears to have a much higher affinity for protoporphyrin – in Saccharomyces, Km is 50 nM [144]. In green plants that synthesize large quantities of chlorophyll, it might prove feasible to divert a much higher proportion of protoporphyrin IX to heme and biliverdin by transfecting them with a constitutively active yeast ferrochelatase, modified to promote its uptake by chloroplasts – thus enabling effective competition with Mg-chelatase activity – while co-transfecting potent heme oxygenase activity. A portion of this biliverdin would then be converted to phytochromobilin. Partial suppression of Mg chelatase activity via antisense DNA or mutation might also promote this goal. In this way, green plants might be turned into rich sources of biliverdin and phytochromobilin. Since the chlorophyll content of spinach is greater than 1% of dry weight, a spinach which made comparable amounts of biliverdin/phytochromobilin could be of considerable utility.

A potential snag is that increased heme levels feed back to suppress synthesis of delta-aminolevulinic acid (ALA) in plants [142]. This problem might be addressed by transfecting a yeast ALA synthetase fused with an amino-terminal signal peptide that directs the enzyme to plastids – as has been accomplished in tobacco plants; [145] the yeast enzyme generates ALA by condensing succinyl-coA and glycine, as in animals. Alternatively, a truncated form of glutamyl-tRNA reductase (rate-limiting for ALA synthesis in plants) not susceptible to inhibition by heme been characterized in barley; [146] transfection of this would presumably render plant heme synthesis less susceptible to feedback control. Maximizing heme oxygenase activity would be expected to moderate the size of the heme pool, and thus lessen feedback inhibition of ALA synthesis; this would also protect plants from the oxidant stress mediated by heme and its precursors.

It may also be feasible to produce commercial quantities of phycobilins in bioengineered bacteria. Recently, Japanese researchers have developed a strain of E. coli transfected with heme oxygenase and bilin reductases – the enzymes required for conversion of heme to phycobilins [147]. Presumably, heme synthesis (and thus phycobilin synthesis) in this organism could be maximized by feeding it ALA while insuring that expression of heme oxygenase was high enough to keep the heme pool low [148].

**Slowing bilirubin conjugation**

There is however a further strategy for exploiting the antioxidant benefits of bilirubin that should be more immediately applicable. Since the hyperbilirubinemia associated with Gilbert syndrome reflects underactivity of hepatic UGT1A1, it may be feasible to induce an "iatrogenic Gilbert syn-
"Iatrogenic Gilbert syndrome"—A strategy for reducing vascular disease with drugs that diminish hepatic glucuronidation activity. In particular, the uricosuric agent probenecid has long been known to have this effect [149–152]—presumably because this drug inhibits transport of UDP-glucuronic acid from the cytoplasm (where it is synthesized) into the endoplasmic reticulum (where UDP-glucuronosyltransferases are located) [153,154]. Indeed, probenecid, in well tolerated clinical doses, is known to prolong the plasma half-life of various drugs whose excretion is partially dependent on glucuronidation. For example, when 500 mg probenecid was administered every 6 h to human volunteers, plasma half-life of acetaminophen increased from 2.5 h to 4.30 h, and 24-h urinary excretion of the glucuronidated metabolite fell by two-thirds [149]. Analogously, lorazepam half-life was increased from 14.3 h to 33.0 h during probenecid administration. Sulfate conjugation of these drugs was not affected. This effect has prompted the suggestion that probenecid be administered as an adjunct to certain types of drug therapy, to boost drug efficacy. Although there do not appear to be any MedLine-traceable reports evaluating the impact of probenecid therapy on serum unconjugated bilirubin—particularly since probenecid is a drug that has been in use for decades and is known to be well tolerated in most subjects.

Other drugs are known to induce hyperbilirubinemia by inhibiting a transporter which expedites hepatocyte uptake of bilirubin. While the bilirubin diacid can pass through membranes fairly readily, its uptake by hepatocytes is accelerated by a membrane transport protein, OATP 1B1, with a high affinity for hydrophobic organic anions [156,157]. Drugs that are hydrophobic anions can compete with bilirubin for access to this carrier, including most notably the anti-tuberculosis antibiotic rifampin. After administration of 900 mg rifampin to 15 healthy subjects, the mean rise in total serum bilirubin 2, 4, and 6 h thereafter was 0.3, 0.5, and 0.6 mg/dl, respectively: that is, serum bilirubin approximately doubled [158]. Not unlikely, co-administration of probenecid and rifampin could achieve serum levels of unconjugated bilirubin comparable to those seen in Gilbert syndrome. A drawback with rifampin is that, is a small proportion of patients, it can have serious side effects, including liver failure in subjects with pre-existing liver disease or who are elderly. Thus, if rifampin were used clinically to raise serum bilirubin, close physician monitoring would be required. It would be desirable to identify another hydrophobic anionic agent—preferably a nutraceutical?—with comparable inhibitory activity for OAT 1B1, but more dependable tolerance.

Finally, agents which competitively inhibit the binding of bilirubin to albumin should be able to increase the access of free unconjugated bilirubin to tissues. Valproate is known to have this effect [159], though its many potential side effects would render it inadvisable for this purpose.

Targeting NADPH oxidase for prevention and therapy

If indeed bilirubin—and possibly also the phycorubin homologues of bilirubin—can decrease the activation and/or activity of NADPH oxidase in many tissues, the ramifications for prevention and therapy may be of stunning breadth, as this enzyme complex appears to be a key mediator of inflammation and hyperplasia in a vast range of pathologies. Increased endothelial NADPH oxidase activity is largely responsible for the oxidant stress-mediated endothelial dysfunction associated with hypercholesterolemia [160–162], hypertension [163–170], hyperglycemia [171,172], insulin resistance syndrome [171,173,174], hyperhomocysteinemia [175,176], elevated C-reactive protein [177], smoking [178,179], and advanced glycation end-products [180,181]—most of the well-characterized vascular risk factors; it also plays an important role in the mediation of ischemia–reperfusion injury [182,183]—consistent with numerous reports that bilirubin is protective in this regard [184–189]. It is also a key mediator of vascular hyperplastic syndromes, including left ventricular hypertrophy [190,191], medial hypertrophy [192,193], and glomerulosclerosis [194–197], as well as the bronchial hyperplasia associated with asthma [95]. And it also plays a role in coagulation and thrombosis—up-regulating platelet aggregation while promoting expression of tissue factor, the trigger for the extrinsic coagulation cascade [198–200]. Oxidants generated by this enzyme complex play a key role in bronchospasm [201] and in mast cell activation [202,203] (note that remission of asthma has been reported during hyperbilirubinemia [204], that bilirubin stabilizes mast cells in vitro [205], and that oral phycocyanin has suppressed allergic reactions in rodents [131]), as well as in the pertur-
Osteoclasts express NADPH oxidase activity, and the hydrogen peroxide that evolves from such activity plays a role in osteoclast differentiation, while also promoting osteoclast-mediated bone resorption [222–226]. Conversely, genetically-linked osteopetrosis, in both humans and mice, is associated with a deficit in NADPH oxidase activity [222,227]. The accelerated bone resorption associated with estrogen deficiency may reflect down-regulation of antioxidant mechanisms that counteract the impact of hydrogen peroxide on osteoclasts [228,229]. Thus, pharmaceutical inhibition of NADPH may have potential for prevention of postmenopausal osteoporosis. Inasmuch as Rac is essential for optimal NADPH oxidase activity in osteoclasts [230], it is tempting to speculate that a decrease in the geranylation of Rac mediates, at least in part, the favorable impact of statins and nitrogen-containing bisphosphonates on bone density [231–236].

Increased NADPH oxidase activity is a mediator of increased mitogenic activity and/or cell survival in various types of cancer; [237–246] moreover, activation of NADPH oxidase plays an obligate role in the process of angiogenesis [247,248]. The NADPH oxidase activity of phagocytes undoubtedly contributes to the increased risk for mutagenesis and cancer associated with chronic inflammation [249–251]. This enzyme is activated in microglia in Alzheimer’s disease [252,253], and appears to mediate the pro-apoptotic impact of amyloid-beta peptide on neurons in this disorder [254]. A possible role for microglial NADPH oxidase activity in the genesis of Parkinson’s disease has also been suggested [255,256].

In obese diabetic KKAY mice, activation of NADPH oxidase in adipocytes has been shown to be a key mediator of the "inflamed" adipocyte phenotype, associated with increased production of TNF-α and other inflammatory cytokines, adipocyte insulin resistance, and the metabolic syndrome. Treatment of such mice with the NADPH oxidase inhibitor apocynin ameliorated the inflamed state of their white adipose tissue, while reducing elevated serum levels of glucose, triglycerides, and insulin [257]. However, Goldstein and colleagues have shown that, in some tissues, including adipocytes, insulin-mediated activation of NADPH plays a role in amplifying insulin signaling, as the derived hydrogen peroxide inhibits PTP-1B, a phosphotyrosine phosphatase that targets the insulin receptor; [258,259] this may be a specific incidence of the more general phenomenon of NADPH oxidase activation up-regulating growth factor signaling. There do not appear to be any studies examining the impact of NADPH oxidase inhibition on insulin function in lean animals. To the extent that failure to inactivate PTP-1B acts as a countervailing negative factor when NADPH oxidase inhibition is employed in insulin resistant or diabetic patients, this effect might be compensated by concurrent administration of cinnamon extract, the insulin-sensitizing non-steroidal anti-inflammatory drug that likewise is induced in these disorders; this peroxynitrite is suspected to mediate much of the neuronal death and dysfunction associated with these syndromes [262]. Moreover, recent evidence suggests that activation of NADPH oxidase within neurons may be a key mediator of excitotoxic neuronal death [263] – a phenomenon that contributes to neuron loss in stroke and neurodegenerative disorders [264,265]. Thus, NADPH oxidase inhibition may have a role to play in the prevention and treatment of various neurodegenerative conditions, possibly including common syndromes such as Alzheimer’s and Parkinson’s diseases [266,252,253,255,267].

Superoxide is emerging as a key mediator of hyperalgesia associated with various chronic pain conditions, and other inflammatory states.
endothelial cells [268–271]. Neuronal NADPH oxidase seems likely to be a prominent source of the superoxide involved in hyperalgesia [272,273]. Indeed, the hyperalgesic impact of nerve growth factor has been traced to NADPH oxidase-generated superoxide, which via p38 MAP kinase activation promotes translation of TRPV1, an ion channel receptor which is a key mediator of nociception [274–276]. Superoxide can also promote activation of c-Src kinase, which boosts TRPV1 activity [277,274]. And, since NO, acting via cGMP, suppresses hyperalgesia [278,279], superoxide’s antagonism of NO bioactivity would be expected to boost pain reception.

Exposure of keratinocytes to UVB triggers activation of NADPH oxidase, which appears to be the chief source of the oxidative stress involved in UVB-mediated skin damage [280,281]. Thus, inhibition of NADPH oxidase may have potential for prevention both of the acute effects of UVB exposure as well as longer term consequences of chronic exposure such as cosmetic skin aging.

Activation of NADPH oxidase appears to play an essential role in the induction of inducible NO synthase triggered by bacterial lipopolysaccharide [282,283]. This may rationalize observations that pre-treatment with hemoglobin (which induces HO-1), bilirubin, or oral phycocyanin prevents circulatory collapse and lethality in rodents subsequently infused with lipopolysaccharide [284–286,132]. Thus, bilirubin and its homologs may have potential for controlling septic shock—a effect recently demonstrated with in bilirubin-in-fused rats [102].

In disorders in which phagocyte hyperactivity plays a pathogenic role, such as emphysema, gastrointestinal ulceration, acute respiratory distress syndrome, and rheumatoid arthritis, partial suppression of phagocytic NADPH oxidase activity may be of benefit [287–289]. Clearly, however, excessive or untimely inhibition of this enzyme could increase the risk for, or severity of, infections; not surprisingly, bilirubin has been shown to impair the bactericidal activity of neutrophils in vitro [290]. However, this effect only became statistically significant at an albumin-bound bilirubin concentration of 150 μM—about fivefold the concentration seen in Gilbert syndrome; thus, moderate elevations of bilirubin may not be harmful in this regard. NADPH oxidase activity plays a role in the expression of MHC class II by antigen-presenting cells [291], rationalizing a recent report that bilirubin inhibits MHC class II expression by endothelial cells [292]. Thus, inhibition of NADPH oxidase activity may influence antigen-specific immune defenses; indeed, biliverdin administration is reported to promote survival of cardiac allografts in rats [117].

Activation of NADPH oxidase may play a prominent role in HIV-1 infection—boosting the transcription of HIV proteins, while also mediating some of the pathogenic consequences of HIV infection. Several HIV proteins—Tat, Nef, and gp120—can stimulate NADPH oxidase activity [293–299]. Since oxidants can up-regulate NF-kappaB activation [300,301], and this transcription factor promotes the transcription of HIV proteins via NF-kappaB response elements in the long terminal repeat (LTR) promoter [302–304], it is reasonable to expect that NADPH oxidase activity will increase the production of HIV particles in infected cells. Of considerable interest in this regard is a recent report that hemin-mediated induction of HO-1 suppresses HIV-1 infectivity in human monocytes; this effect reflects, at least in part, inhibition of Tat-mediated activation of the LTR promoter [305]. Also of interest is an older report that both bilirubin and biliverdin can act as HIV protease inhibitors in 1 μM concentrations [306]. Activation of NADPH oxidase may also mediate some of the pathogenicity of HIV-1 infection. Thus, exposure of neurons to gp120 leads to ceramide-mediated apoptosis; this generation of ceramide, in turn, reflects NADPH oxidase-mediated oxidant stress triggered by interaction of gp120 with neuronal CXCR4 receptors [298]. It is thought that this mechanism may play a role in AIDS-related dementia. Peroxynitrite production by HIV-infected microglia might also contribute in this regard [307] — a phenomenon that likewise is dependent on NADPH oxidase activation. With respect to the possibility of targeting NADPH oxidase in the management of HIV infection, however, it should be noted that the immunosuppressive potential of such a strategy might make its use problematical in patients who have progressed to frank immunodeficiency.

It should be noted that various isoforms of NADPH oxidase are expressed in different tissues; in particular, the gp91phox subunit (now known as Nox2) found in phagocytes is replaced by other members of the Nox family in other cell types, and functionally distinct homologues of p47phox and p67phox are also expressed [308–311]. In addition, activation mechanisms for NADPH oxidase may vary from tissue to tissue. Thus, it is quite conceivable that bilirubin and bilirubin homologues are not equally active in suppressing the activity (or activation) of all sub-types of NADPH oxidase. In particular, the neutrophil study cited above sug-
gests that possibility that the high-capacity NADPH oxidase expressed by phagocytes is less susceptible to inhibition than are some lower capacity forms expressed in other tissues. Conceivably, the molecular target of bilirubin in the NADPH complex could be present in functional excess in phagocytes, such that higher concentrations of bilirubin would be required to achieve meaningful inhibition. If indeed phagocytic NADPH oxidase is less sensitive to inhibition by bilirubin, this might lessen the risk associated with bilirubin-elevating therapies, but clearly might also diminish the scope of applicability of such therapies.

It is increasingly evident that activation of NADPH oxidase performs a signaling function in a diverse range of non-phagocytic cells; presumably, the hydrogen peroxide and peroxynitrite derived from this enzyme complex induce reversible oxidation of sulphydryl groups in signaling intermediates, such that these intermediates become more effective for signal transduction. But excessive activation of NADPH oxidase can subject cells to harmful oxidant stress, or lead to maladaptive cellular hyperactivity. Subsequent oxidant-mediated induction of HO-1, by generating the NADPH oxidase inhibitors bilirubin and CO, provides homeostatically appropriate feedback control of this enzyme complex. In pro-inflammatory disorders in which NADPH oxidase is chronically up-regulated, it may prove possible to mimic or amplify this feeding mechanisms for oxidant generation.

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References


[56] Naylor LH, Clark EM, d(T)g.in(d(C)AN sequences upstream of the rat prolactin gene form Z-DNA and inhibit gene transcription. Nucleic Acids Res 1990;18:1595–601.


103 Kwak JY, Takeshige K, Cheung BS, Minakami S. Bilirubin inhibits the activation of superoxide-producing NADPH oxidase in a neutrophil cell-free system. Biochim Biophys Acta 1997;1315:207–13.


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