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Antioxidant bilirubin works in multiple ways to reduce risk for obesity and its health complications

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UNCONJUGATED BILIRUBIN FUNCTIONS PHYSIOLOGICALLY TO INHIBIT NADPH OXIDASE COMPLEXES

Gilbert syndrome (GS) is a common genetic variant in which plasma unconjugated bilirubin levels are elevated throughout life, in the absence of hepatic pathology.[1] This typically reflects decreased hepatic capacity for conjugation of bilirubin coupled with an upregulation of bilirubin generation. Typically, subjects with GS are homozygous for promoter mutations compromising transcriptional efficiency in the gene coding for uridine-diphosphoglucuronate glucuronosyltransferase 1A1 (UGT1A1), which links bilirubin to glucuronic acid; as a result, hepatic expression of this enzyme is decreased, although the enzyme itself is functionally normal. However, plasma bilirubin levels in many people homozygous for such mutations fail to exceed the level (defined as either 17.1 or 20 µmol/L) considered diagnostic for GS. Hence, subjects with GS also are characterised by an increased rate of bilirubin generation, ultimately traceable to increased heme synthesis. In some cases, this may reflect upregulated heme oxygenase activity, which would reflexly boost heme production.[1 2]

Epidemiological studies have found that GS confers potent and versatile health protection.[2–5] Notably, an analysis of the Health Improvement Network primary care database in the UK found that, after adjustment for pertinent covariants, a diagnosis of GS was associated with a relative risk for all-cause mortality of 0.5 (95% CI 0.4 to 0.7; p<0.001).[6]

This remarkable health benefit appears likely to stem largely from the fact that physiological intracellular levels of unconjugated bilirubin inhibit certain common isoforms of NADPH oxidase.[7–11] These membrane-bound superoxide-generating complexes are a major source of the oxidants that drive or exacerbate a high proportion of health disorders. Bilirubin's inhibitory impact on NADPH oxidase activity presumably explains much of the profound antioxidant activity of heme oxygenase, which cleaves heme to yield biliverdin, carbon monoxide and free iron; biliverdin is then rapidly reduced by the ubiquitously expressed enzyme biliverdin reductase to yield bilirubin. Expression of inducible form of heme oxygenase, HO-1, can be boosted by oxidative stress—often derived from NADPH oxidase activity; the resultant production of bilirubin feeds back to quell this oxidative stress.[10] Although bilirubin can also act as a direct oxidant scavenger, its physiological intracellular level—in the low nanomolar range—is too low to compete in this regard with other intracellular scavengers (eg, glutathione, ascorbate) present in millimolar concentrations.[3]

While there currently is a common perception among medical scientists, rooted in the disappointing results of clinical trials with nutritional scavenging antioxidants such as ascorbate, alpha-tocopherol and beta-carotene, that antioxidants have limited potential for conferring health protection, this perception fails to grasp the crucial difference between scavenging antioxidants and 'source antioxidants', of which bilirubin is a key example.[12] Source antioxidants, by definition, prevent oxidant production by suppressing superoxide generation at its source; they therefore oppose the often proinflammatory effects of hydrogen peroxide on cellular signalling, and prevent conversion of (often protective) nitric oxide to the potent oxidant peroxynitrite[13 14]—effects which scavenging antioxidants cannot achieve. Statins and angiotensin II antagonist drugs likewise can function as source antioxidants in vascular

tissues by inhibiting the activation of certain NADPH oxidase complexes.[12 15 16]

MIMICKING GS AS A CLINICAL ANTIOXIDANT STRATEGY

Although oral administration of bilirubin as a clinical strategy is not feasible, owing to its extremely low water solubility, plasma and tissue bilirubin levels can be increased by drugs or nutraceuticals that inhibit UGT1A1; this approach has been dubbed 'iatrogenic Gilbert syndrome'.[3 17] Alternatively, bilirubin's much more soluble precursor biliverdin could be administered orally.[18] While biliverdin is a complex molecular quite expensive to synthesise, and no rich natural sources exist, the biliverdin metabolite phycocyanobilin (PhyCB) functions as a light-harvesting chromophore in cyanobacteria and certain blue-green algae, such as the food spirulina; PhyCB can constitute 0.6% or more of the dry weight of spirulina.[19] PhyCB is readily converted by biliverdin reductase to the bilirubin analogue phycocyanorubin, which appears to share bilirubin's ability to inhibit NADPH oxidase complexes.[19–21] Arguably, this may largely explain the versatile antioxidant and anti-inflammatory properties of oral spirulina (or of phycocyanin, the spirulina protein to which PhyCB is covalently attached) in rodent studies.[19 22 23] Hence, spirulina—or, preferably, spirulina extracts enriched in PhyCB, since spirulina itself has an unappetising flavour and odour—may have considerable potential as source antioxidants.

GS IS ASSOCIATED WITH LOWER GAIN IN FAT MASS DURING LATER LIFE

A recent cross-sectional epidemiological study evaluating subjects with GS has discovered that GS is associated with a reduced tendency to gain body fat in later life.[24] The study enrolled 124 subjects with GS (average plasma unconjugated bilirubin 30.7 µM) and 124 age-matched and gender-matched controls (8.7 µM). Unlike previous studies evaluating Body Mass Index (BMI) in subjects with GS—which found modestly lower BMI as compared with age-matched controls[25]—this study was designed to analyse young subjects (under 35) and older subjects (over 35) separately. Whereas, as compared with age/gender-matched controls, the subjects with GS in the younger group were found to have a modestly but significantly lower BMI (22.5 vs 23.5), the disparity for the older group was dramatic—23.8 vs 27.2 ($p<0.001$). Moreover, the average body fat content in the older GS group was 21.8%, as compared with 29.3% in their controls ($p<0.01$). A reasonable deduction is that chronically elevated free unconjugated bilirubin—and perhaps an upregulation in intracellular bilirubin generation—somehow opposes age-related gain in body fat.

Of related interest is a study showing that intraperitoneal administration of bilirubin—administered daily for 14 days—inhibits weight gain in rats fed a diet high in fats and sugar.[26] Bilirubin injections were also found to prevent deterioration of glucose tolerance. A trend towards decreased calorie consumption in the bilirubin-treated rats just failed to achieve statistical significance ($p=0.06$).

A credible case can be made that the favourable impact of elevated bilirubin on risk for undesirable weight gain reflects preservation of hypothalamic leptin sensitivity.[27] Rodents rendered obese with high-fat, high-sugar 'Western' diets develop leptin resistance in the arcuate nucleus of the hypothalamus; for this reason, as the rodents become obese, the evolving hyperleptinemia fails to oppose the hyperphagia induced by such diets.[28–30] This leptin resistance is mediated, at least in part, by activation and proliferation of microglia in the arcuate nucleus, which can produce cytokines (notably tumour necrosis factor-α[31] that counteract leptin signalling).[32–34] Activation of NADPH oxidase is a key mediator of pro-inflammatory microglial activation[35–37]; hence, elevated bilirubin might be expected to support effective leptin function in the arcuate nucleus, thereby aiding appetite control.[27]

BILIRUBIN ACTS ON ADIPOCYTES TO COUNTER METABOLIC SYNDROME

Oxidative stress in adipocytes, stemming largely from NADPH oxidase activity, appears to play a key role in the induction of insulin resistance and the skewing of adipokine and cytokine production in hypertrophied adipocytes.[38–43] Hence, bilirubin and heme oxygenase activity could be expected to aid maintenance of adipocyte insulin sensitivity. Indeed, plasma levels of unconjugated bilirubin have been found to correlate inversely with risk for metabolic syndrome and diabetes in prospective epidemiological studies, as confirmed in a recent meta-analysis.[44]

In both cross-sectional and prospective studies, higher plasma bilirubin levels are associated with better insulin sensitivity and decreased risk for metabolic syndrome and type 2 diabetes—independent of BMI.[45 46] Hence, bilirubin may function both to prevent adipocyte hypertrophy—via its hypothalamic effects—and to improve the function of adipocytes that have already hypertrophied.

A direct protective effect of bilirubin on adipocyte function may be largely responsible for this phenomenon. Hypertrophied insulin-resistant adipocytes are characterised by increased oxidative stress, derived in large part from NADPH oxidase complexes (Nox2 and Nox4 dependent); the activated macrophages in hypertrophic visceral adipose tissue (expressing Nox2) can also contribute to this oxidant load.[39–41 47] There is considerable evidence that this oxidant stress plays a mediated role in the insulin resistance, upregulation of proinflammatory cytokines and diminished adiponectin secretion characteristic of hypertrophied visceral adipocytes.

Adverse effects of oxidants on adipocyte function may be mediated in large part by increased activation of the ASK1-MKK4-JNK signalling pathway.[48] Activation of JNK in hypertrophied adipocytes plays a central role in

metabolic syndrome.[49 50] Adipocyte-specific expression of dominant-negative JNK prevents systemic insulin resistance, glucose intolerance and hepatic steatosis in mice fed a high-fat diet.[50] Moreover, this prevents the decline in adiponectin production, the increase in pro-inflammatory cytokines and the infiltration of activated macrophages seen in the adipose tissue of mice fed such a diet. Activated JNK impairs insulin signalling by phosphorylation of Ser307 in insulin receptor substrate-1 (IRS-1), preventing it from interacting with the activated insulin receptor and accelerating its proteasomal degradation.[51] JNK may promote adipocyte expression of pro-inflammatory cytokines such as TNFα and MCP-1, at least in part, by phosphorylating c-Jun and thereby boosting AP-1 transcriptional activity.[52–54] Phosphorylation of PPARγ by JNK diminishes its transcriptional activity, and this might account for JNK's ability to suppress expression of adiponectin, a hormone crucial for the maintenance of hepatic insulin sensitivity, control of gluconeogenesis and prevention of hepatic steatosis.[55–59] And JNK mediates the suppressive effect of TNFα on β-klotho expression in adipocytes, an effect responsible for the decreased responsiveness of adipocytes to fibroblast growth factor-21, a key stimulant to adiponectin expression.[60]

ASK1 is a prominent upstream activator of JNK (via MKK4) in adipocytes; its expression is upregulated in the visceral adipocytes of obese patients.[61] The kinase activity of ASK1 is inhibited by its binding with the redox-modulated protein thioredoxin.[62 63] Oxidants such as hydrogen peroxide alter the structure of this protein by inducing formation of an intramolecular disulfide bridge; this structural alteration prevents the binding of thioredoxin to ASK1.[64 65] The reduced structure of thioredoxin is restored by thioredoxin reductase activity.[65] When oxidant production frees ASK1 from its interaction with thioredoxin, ASK1 is susceptible to activation by signalling complexes formed after activation of toll receptor 4 (TLR4) or TNFα.[63] These complexes contain TRAF6 and TRAF2, respectively, which can bind with free ASK1 in such a way as to promote its homodimerisation, thereby unleashing its kinase activity.[63 66] Activation of TLR4—via binding to a saturated fatty acid/fetuin A complex[67]—as well as TNFα-mediated activation of its receptor on adipocytes, are prominent mechanisms for induction of insulin resistance in hypertrophied adipocytes.[68] Activation of these receptors also boosts oxidant production, a prerequisite for their activation of ASK1.[63 69] Suppression of this oxidant production, as by bilirubin or bilirubin mimesis, can therefore be expected to blunt activation of the ASK1-MEK4-JNK signalling pathway that is a crucial mediator of insulin resistance syndrome.

Consistent with this prediction, adipocyte-specific overexpression of HO-1, systemic induction HO-1, and oral administration of phycocyanin or whole spirulina have all been shown to promote proper adipocyte function and mitigate induction of metabolic syndrome in rodents fed high-fat or fructose-rich diets.[70–75] Additionally, treatment with the broad-spectrum NADPH oxidase inhibitor apocynin attenuated development of metabolic syndrome in KKAy diabetes-prone mice. Oral administration of phycocyanin has a similar impact in these mice.[76] The favourable impact of high-dose spirulina on insulin sensitivity in treated patients with HIV has been noted.[77]

BILIRUBIN DIRECTLY PROTECTS TISSUES TARGETED BY METABOLIC SYNDROME

Optimal adipocyte function helps to prevent hepatic steatosis by moderating free fatty acid flux to the liver when insulin is elevated and also by enabling appropriate adiponectin production. It is therefore notable that dietary spirulina has been found to decrease liver fat content and mitigate liver inflammation in rodent models of metabolic syndrome.[78–80] Moreover, two open clinical trials in which spirulina was administered orally in centrally obese patients with non-alcoholic fatty liver disease have reported improved liver function (decreased serum hepatic enzymes and/or decrease liver fat as assessed by sonography).[81 82] In one of these trials, the patients ingested 6 g spirulina daily for 6 months; at the end of the study, liver enzymes had dropped by about a third, serum lipid profile had improved (lower triglycerides and LDL-C, higher HDL-C), insulin resistance assessed by HOMA had dropped by 20% and BMI had dropped by 8%—changes that were all highly significant.[82] Since the patients had not been asked to modify their diets or exercise habits, the observed weight loss conceivably could be attributable to an improvement in hypothalamic inflammation. Suppression of oxidant and cytokine production by infiltrating macrophages and Kupffer cells might also contribute to the improvement in liver function seen during spirulina administration, as activation of NADPH oxidase in these cells promotes the oxidant stress and inflammation that collaborates with lipid overload in induction of steatohepatitis.[83–85] Moreover, activation of NADPH oxidase in stellate cells is a driver of the liver fibrosis that leads to end-stage cirrhosis.[86]

Metabolic syndrome also impairs vascular endothelial function—a likely reason why this syndrome collaborates with elevated low-density lipoprotein to promote atherogenesis and vascular events.[87–89] Increased exposure to free fatty acids and proinflammatory cytokines, as well as decreased adiponectin levels, may play a role in mediating this endothelial dysfunction. Increased vascular expression and activity of NADPH oxidase is a feature of metabolic syndrome and a mediator of the associated endothelial dysfunction.[90–93] Ingestion of spirulina or phycocyanin has been found to exert anti-atherosclerotic effects in rodent models.[94–97]

Prospective epidemiology links higher plasma bilirubin with lower risk for myocardial infarction, stroke, left ventricular hypertrophy (LVH) and heart failure, all of which are more common in those with metabolic syndrome.[98–101] Superior endothelial function may underlie much of this apparent protection, whereas

diminished platelet aggregability may contribute to lower risk for vascular events, and better cardiac nitric oxide bioactivity stemming from control of NADPH oxidase-mediated oxidative stress may play a role in the diminished risk for LVH and heart failure associated with increased bilirubin.[102–107]

BILIRUBIN AIDS PREVENTION OF TYPE 2 DIABETES AND OF DIABETIC COMPLICATIONS

In pancreatic beta cells of patients with metabolic syndrome, an increase in Nox2-dependent oxidant production induced by joint overexposure to free fatty acids and glucose (glucolipotoxicity) can lead to a failure of glucose-stimulated insulin release and an upregulation of apoptosis that ushers in type 2 diabetes—which in turn sustains and exacerbates the glucolipotoxicity mediating beta-cell failure.[108–112] This phenomenon may contribute to the inverse correlation of serum bilirubin with risk for type 2 diabetes. Indeed, oral biliverdin postpones onset of diabetes in db/db mice.[113] Moreover, hyperglycaemia-induced NADPH oxidase activation is a mediator of the microvascular complications of diabetes; in diabetics, serum bilirubin correlates inversely with risk for diabetic nephropathy, retinopathy and neuropathy.[114–118] Most strikingly, when diabetics with documented GS were matched with other diabetics, not known to have GS, with respect to age, sex, duration of diabetes and severity of hyperglycaemia, those with GS were found to be less than a third as likely to develop nephropathy, retinopathy or coronary disease.[119] Moreover, oral administration of biliverdin or of phycocyanin has been shown to prevent glomerulosclerosis in db/db mice.[18 21]

Hence, bilirubin or bilirubin mimesis may be beneficial with respect to the hypothalamic inflammation that promotes inappropriate weight gain, the adipocyte dysfunction that leads to metabolic syndrome once obesity has emerged, the hepatic and vascular pathology that often accompanies metabolic syndrome, the glucolipotoxicity-induced beta-cell dysfunction that can precipitate onset of type 2 diabetes in patients with metabolic syndrome, and the microvascular and macrovascular complications of diabetes. Moderate and safe downregulation of NADPH oxidase activity may thus have profound implications for preservation of metabolic and vascular health.

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