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2 ‘‘Iatrogenic Gilbert syndrome’’ – A strategy for 3 reducing vascular and cancer risk by increasing 4 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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35 Antioxidant role of heme oxygenase

36 Heme oxygenase has emerged in recent research
37 as an antioxidant enzyme of great physiological
38 importance [1,2]. There are at least three iso-
39 forms of this enzyme in mammals; two of these
40 are constitutively expressed, but heme oxygenase-1,
41 the most important from the standpoint of
42 antioxidant protection, is inducible. Heme oxygenase
43 functions to cleave heme, converting it to carbon
44 monoxide, free ferrous iron, and the linear tetrapyrrole
45 biliverdin; the latter is rapidly reduced to bilirubin
46 by the ubiquitously expressed biliverdin reductase.
47 Heme is synthesized in all nucleated cells, reflecting
48 the fact that this crucial cofactor is not exchanged
49 between tissues. Heme oxygenase activity promotes
50 recycling of tissue iron; when tissue heme levels
51 increase, heme interacts with the inhibitory transcrip-
52 tion factor Bach1, disinhibiting HO-1 transcription
53 and thus promoting heme catabolism [3,4]. This effect
54 is also protective, since elevated heme levels can
55 induce oxidant stress. On the other hand, since
56 heme plays a central role in aerobic energy metabo-
57 lism, hypoxia promotes increased production of
58 Bach1, suppressing HO-1 transcription and thereby
59 conserving heme. The antioxidant function of HO-1
60 is primarily attributable to the fact that bilirubin is
61 a potent and highly versatile antioxidant, scavenging
62 superoxide, peroxy radicals, hydroxyl radicals, hypo-
63 chlorous acid, and singlet oxygen, as well as the
64 reactive nitrogen species nitroxyl and peroxynitrite;
65 [5–10] furthermore, bilirubin can reduce the α -
66 tocopheroxyl radical, thereby promoting recycling
67 of vitamin E [10]. This presumably explains why
68 the HO-1 promoter has evolved to respond to oxidant
69 stress via antioxidant response elements; the transcrip-
70 tion factor Nrf2, whose half-life and nuclear access
71 is increased by oxidants and electrophiles that covalently
72 modify cysteine residues in its binding protein Keap1
73 [11,12], competes with Bach1 for heterodimerization
74 with Maf transcription factors, producing heterodimers
75 which boost HO-1 transcription by binding to multiple
76 antioxidant response elements (also known as “Maf
77 recognition elements”) in the HO-1 promoter [3].
78 Phase II inducer chemicals, as well as a wide variety
79 of agents which promote or mediate oxidant stress –
80 cytokines, endotoxin, hyperoxia, ultraviolet light,
81 X-irradiation, hydrogen peroxide, peroxynitrite,
82 taurine chloramine, phorbol esters – have been
83 shown to act as HO-1 inducers [13–15]. Other
84 agents reported to induce HO-1 in various types
85 of cells include cGMP, statins, alanine, probucol,
86 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- κ B,
thereby exerting anti-inflammatory effects [23].
Moreover, like nitric oxide (NO), it can activate the
soluble guanylate cyclase [24]; however, since it
increases the V_{max} 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 expres-
sion 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-
ing 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 an enzymatic reductive
mechanism [34,35]. Consistent with this view,
this group has shown that inhibition of biliverdin
reductase with

146 small interfering RNA markedly boosts oxidant lev- 199
147 els in cells – indeed, to a greater degree than when 200
148 glutathione synthesis is blocked. They also report 201
149 that as little as 10 nM of albumin-bound bilirubin 202
150 can protect cells from 100 μM hydrogen peroxide 203
151 – an effect which presumably demonstrates the rap- 204
152 id turnover of the bilirubin–biliverdin cycle. This 205
153 hypothesis provides a satisfying rationale for the 206
154 otherwise rather inexplicable fact that mammals 207
155 go to the trouble and energetic expense of convert- 208
156 ing biliverdin – a soluble, readily excreted com- 209
157 pound with antioxidant activity of its own – to 210
158 poorly soluble and potentially toxic bilirubin. None- 211
159 theless, Stocker emphasizes that not all reactions 212
160 between bilirubin and oxidants generate biliverdin 213
161 [10] – as in the case of peroxy radicals, for exam- 214
162 ple [5]. On the other hand, Kaur et al., report pro- 215
163 duction of biliverdin when bilirubin reacts with 216
164 active nitrogen species, and comment approvingly 217
165 on the Snyder thesis [9]. 218

166 Protective potential of HO-1 confirmed

167 The likelihood that HO-1 activity has an important 219
168 impact on the pathogenesis of various diseases 220
169 finds strong support in rodent studies as well as in 221
170 epidemiology. Increasing tissue activity of HO-1, 222
171 either by transgenic methods or by treatment with 223
172 HO-1 inducers, has protected rodents from athero- 224
173 genesis, thrombosis, renal, hepatic, or pulmonary 225
174 injury, ischemia–reperfusion injury, and angioten- 226
175 sin II-mediated cardiac hypertrophy; in many of 227
176 these studies, concurrent administration of HO-1 228
177 inhibitors abrogated this protection [36–54]. Con- 229
178 versely, HO-1 knockout mice are more prone to 230
179 atherogenesis and vein graft stenosis and calcifica- 231
180 tion [55]. HO-1 activity appears to be of particular 232
181 importance in endothelial cells; increased endo- 233
182 thelial HO-1 activity suppresses induction of adhe- 234
183 sion factors and monocyte transmigration by 235
184 cytokines, oxidants, and oxidized LDL, prevents 236
185 the sloughing of endothelial cells in diabetic rats, 237
186 and diminishes angiotensin II-mediated DNA dam- 238
187 age [29,31,33,56–59]. Indeed, it may not be exag- 239
188 gerating to state that, in rodents, up-regulation of 240
189 HO-1 activity has been found to confer protection 241
190 in virtually every type of free radical-mediated 242
191 pathology in which it has been studied – a tribute 243
192 to the potency and versatility of the antioxidant 244
193 activity of bilirubin (albeit CO has contributed to 245
194 the observed protection in some instances). 246

195 The impact of HO-1 expression on human 247
196 pathologies can be readily assessed through epide- 248
197 miology, thanks to the fact that a common poly- 249
198 morphism in the promoter region of the HO-1

gene has a notable impact on gene expression. 250
In humans, this promoter contains a GT dinucleo- 251
tide repeat of variable length (GT)_n. The most 252
commonly encountered lengths are of 23 and 30 253
repeats. The expression of genes containing 25
or fewer repeats has been found to be greater 254
than that of genes containing over 25 repeats 255
[60–62], presumably because DNA with a greater 256
number of GT repeats can twist into a "Z" config- 257
uration that is unfavorable for transcription [63]. 258
Epidemiologists have thus stratified subjects into 259
those with a small (S) repeat promoter – associ- 260
ated with increased expression of HO-1 – and 261
those with a large (L) repeat promoter, and have 262
then assessed the frequency of these genes types 263
in subjects afflicted with various pathologies. Per- 264
haps not surprisingly, the L type gene has been 265
found to be relatively more common in cases than 266
controls in studies examining risk for emphysema 267
(in smokers), coronary artery disease, abdominal 268
aortic aneurysms, restenosis after coronary stent- 269
ing or peripheral angioplasty, kidney allograft fail- 270
ure, oral squamous cell carcinoma in betel nut 271
chewers, and lung adenocarcinoma in male smok- 272
ers [3,64,65]. To date, no such relationship has 273
been seen with respect to myocardial infarction, 274
Alzheimers, or Parkinson disease [60,66]. Re- 275
search of this type is still in an early stage, so 276
many more relevant findings can be expected in 277
the near future. 278

Perhaps the most intriguing such study focused 279
on longevity in the Japanese population. Sasaki 280
and colleagues determined the frequency of S, M 281
(mid-size repeats) and L alleles as a function of 282
age in 512 healthy subjects [67] In males, the fre- 283
quency of the class L allele (defined as genotype 284
L/L, L/M, or L/S) declined significantly and mark- 285
edly as a function of age (<60y, 23%; 60–74y, 286
16%; ≥75y, 10%). In women, the decline did not 287
achieve significance (<60y, 22%; 60–74, 22%; 288
≥75y, 15%). (Arguably, the trend in the women 289
might have achieved significance if a higher age 290
bracket had been included in the analysis; the 291
average lifespan of Japanese women is now 86 292
years, and little enrichment of the higher expres- 293
sion alleles could be expected until an ample frac- 294
tion of the women had died.) These findings thus 295
suggest that up-regulation of HO-1 activity may 296
provide protection from all-cause mortality. 297

248 Prognostic significance of serum bilirubin 249

A related strain of epidemiological research has at- 250
tempted to correlate serum bilirubin levels with 251

252 endpoints related to atherosclerosis – coronary or
253 carotid stenosis, ischemic heart disease, and
254 cardiovascular death. The first such study, by
255 Schwertner and colleagues, examined the preva-
256 lence of subclinical coronary stenosis ($\geq 50\%$) as a
257 function of bilirubin level; a marked inverse associ-
258 ation was noted. Relative risk in the four quartiles
259 of serum bilirubin was found to be 1.0, 0.6, 0.4,
260 and 0.3 [68]. The striking findings of this study
261 encouraged a number of other groups to undertake
262 analogous investigations. A sufficient number of
263 such studies have become available to enable a re-
264 cent meta-analysis by Novotny and Vitek, incorpo-
265 rating 11 suitable studies, of both cross-sectional
266 and prospective design [69]. The authors conclude
267 that “a close negative relationship was found
268 between serum bilirubin levels and severity of ath-
269 erosclerosis (Spearman rank coefficient $r = -0.31$,
270 $P < 0.0001$). Unambiguous inverse relationship be-
271 tween serum bilirubin levels and atherosclerosis
272 was demonstrated in this preliminary meta-
273 analytic study.” In dealing with a study that had
274 demonstrated a U-shaped relationship between
275 serum bilirubin and ischemic heart disease inci-
276 dence (i.e. higher risk with high bilirubin) [70],
277 the authors showed that the data from this study
278 fit the overall pattern if patients experiencing liver
279 failure (elevated liver enzymes) were excluded
280 from the analysis.

281 In light of the fact that oxidants are prominent
282 mediators of “spontaneous” mutagenesis, a few
283 studies have examined the relationship of HO-1
284 polymorphisms or of serum bilirubin to cancer risk.
285 In a prospective Belgian study, comparing the high-
286 est ($\geq 6\text{mg/L}$) vs. the lowest ($\leq 2\text{mg/L}$) categories
287 of serum bilirubin, relative risk of cancer mortality
288 was 0.42 for men (significant) and 0.76 for women
289 (non-significant) [71]. (Surprisingly, bilirubin did
290 not predict cardiovascular mortality in this study,
291 whose findings were incorporated into the meta-
292 analysis cited above.) In a study utilizing data from
293 the Third National Health and Nutrition Examina-
294 tion Survey, an increase of serum bilirubin of
295 1 mg per dl was associated with a relative risk of
296 prevalent non-dermatological cancer of 0.81 and
297 of prevalent colon cancer of 0.257 (95% CI 0.254–
298 0.260); the authors postulated that enterohepatic
299 circulation of bilirubin, exposing colonic mucosa
300 to relatively high levels, accounted for the unusu-
301 ally strong seeming impact on colon cancer risk
302 [72]. On the other hand, a previous report saw no
303 such association between bilirubin and colon can-
304 cer risk [73]. In a case–control study of breast can-
305 cer, risk associated with serum bilirubin in the
306 upper quartile, relative to the lower quartile, was
307 0.50 (95% CI 0.26, 0.97) [74]. Finally, as noted

308 above, the high expression form of HO-1 has been
309 associated with reduced risk for squamous oral can-
310 cer in betel nut chewers [75]. Thus, although rele-
311 vant data are still somewhat sparse, the few
312 studies that exist are encouraging in regard to the
313 possible impact of bilirubin on cancer risk.

314 With respect to studies correlating increased
315 serum bilirubin with better health outcomes, it is
316 important to ask: what is the significance of ele-
317 vated bilirubin? Is it merely serving as a marker
318 for increased HO-1 activity in tissues, associated
319 with an increased whole-body production rate of
320 heme and bilirubin? Or do these correlations also
321 reflect the fact that increased serum bilirubin –
322 possibly stemming from decreased efficiency of hep-
323 atic conjugation and excretion of this antioxidant,
324 or from increased erythrocyte turnover – can boost
325 tissue levels of bilirubin sufficiently to provide
326 important antioxidant protection? While the latter
327 may seem intuitively obvious, it should be noted
328 that serum bilirubin, because of its poor solubility,
329 is almost wholly bound to albumin; only a very tiny
330 fraction of unconjugated plasma bilirubin is in free
331 form. Indeed, it previously was thought that only
332 0.005% of total plasma unconjugated bilirubin was
333 unbound [76]; this would imply a free plasma biliru-
334 bin level so low that its ability to influence tissue
335 bilirubin levels meaningfully would be doubtful.
336 However, earlier studies did not account for the
337 fact that affinity of albumin for bilirubin declines
338 substantially as albumin concentration increases.
339 More recent work suggests that, at a physiological
340 adult albumin concentration of $600\ \mu\text{M}$, the affinity
341 constant for bilirubin is approximately $2.3 \times$
342 $10^6\ \text{L mol}^{-1}$ [77,78]; this implies that, at a typical
343 adult serum unconjugated bilirubin concentration
344 of $10\ \mu\text{M}$, the concentration of unbound bilirubin
345 will be about 7.4 nM, a level which is meaningful
346 relative to the tissue level of 10–50 nM (much of
347 it presumably associated with membranes or
348 hydrophobic sites on proteins) reported by Snyder
349 et al [35]. In the diacid form which predominates
350 at physiological pH, free bilirubin readily passes
351 through membranes to enter or exit cells [78,79].
352 And there can be no doubt that, at pathological
353 plasma levels ($>200\ \mu\text{M}$), plasma bilirubin can raise
354 tissue levels sufficiently to give rise to kernicterus
355 in newborns.

356 An alternative or adjunctive possibility is that
357 decreased serum bilirubin may be a marker for a
358 metabolic state that promotes vascular disease.
359 Several cross-sectional studies have found that
360 serum bilirubin correlates inversely with risk fac-
361 tors associated with insulin resistance syndrome,
362 including serum insulin, triglycerides, systolic blood
363 pressure, apolipoprotein B, and adiposity [80–82].

364 Do elevated insulin levels influence bilirubin metab- 420
365 olism, or, conversely, do elevated bilirubin levels 421
366 promote insulin sensitivity and discourage weight 422
367 gain? In this regard, insulin can decrease expression 423
368 of delta-aminolevulinic acid synthase – the rate- 424
369 limiting enzyme for heme production – in hepato- 425
370 cytes [83]; whether it does so in other tissues (most 426
371 heme is synthesized in bone marrow erythroblasts) 427
372 is not known. Even if insulin resistance syndrome 428
373 does somehow suppress serum bilirubin, this is unli- 429
374 kely to provide the entire explanation for the asso- 430
375 ciation of low bilirubin with elevated coronary risk, 431
376 as many studies found that this association per- 432
377 sisted after statistical adjustments for known car- 433
378 diovascular risk factors. A similar comment could 434
379 be made with respect to evidence that smokers 435
380 tend to have decreased bilirubin levels [80,81]. 436

381 Data pertaining to Gilbert syndrome strongly 437
382 suggest that moderately increased plasma free bil- 438
383 irubin can indeed provide important antioxidant 439
384 protection for tissues. Gilbert syndrome is an 440
385 innocuous recessive genetic variant in which hepa- 441
386 tocytes express decreased amounts (~30% of nor- 442
387 mal) of UDP-glucuronosyltransferase type 1A1 443
388 (UGT1A1), the enzyme almost solely responsible 444
389 for bilirubin conjugation; the mutation is in the 445
390 promoter region, leading to decreased expression 446
391 of an enzyme with normal structure and specific 447
392 activity [84]. As a result, serum unconjugated bil- 448
393 irubin tends to be elevated by 2–3-fold; serum bil- 449
394 irubin is typically about 30 μM in Gilbert subjects. 450
395 (No known adverse effects are associated with Gil- 451
396 bert syndrome, notably distinguishing it from type 452
397 1 Crigler–Najjar syndrome, in which UGT1A1 activ- 453
398 ity is absent owing to a mutant enzyme, and serum 454
399 bilirubin is an order of magnitude higher, giving rise 455
400 to kernicterus and other severe complications.) 456
401 [85] Czech researchers recently recruited 50 sub- 457
402 jects with Gilbert syndrome, of age 40 and older; 458
403 average age of the group was 50; they observed 459
404 that only one member of this group – 2% (0.05– 460
405 10.7%, 95% CI) – had symptomatic ischemic heart 461
406 disease [86] In contrast, prevalence of coronary 462
407 disease in an age- and sex-matched control group 463
408 was found to be 12%, and the authors state that 464
409 prevalence of coronary disease in this age group 465
410 among Czech subjects, as described in the litera- 466
411 ture, tends to be 10–20%. Moreover, this one case 467
412 of coronary disease appeared to be atypical, in that 468
413 neither the coronary nor carotid arteries were 469
414 detectibly stenotic. Perhaps more astonishing was 470
415 the fact that only 1 of the 50 Gilbert subjects 471
416 was hypertensive. While it would be rash to read 472
417 too much into one small study – attempts to con-
418 firm this finding in other populations should be an
419 urgent priority – these results, if confirmable, im-

ply 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 affor-
ded by up-regulation of HO-1 activity in rodent
studies, this conclusion is at least reasonably
credible.

The Gunn rat is characterized by hyperbilirubi-
nemia attributable to a complete absence of
UGT1A1 activity; it may thus may 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 im-
pact of angiotensin II infusion on blood pressure
and endothelial function [87]. In particular, the
ability of angiotensin II to raise the endothelial
dihydrobiopterin/tetrahydrobiopterin ratio –
thereby compromising NO synthase activity – is vir-
tually abolished.

Why is bilirubin such an important anti-oxidant?

Albumin-bound bilirubin makes a substantial con-
tribution to plasma antioxidant activity [88]. Biliru-
bin is bound in such a way that the C-10 methylene
group can readily donate electrons to plasma oxi-
dants [10]; thus, it may help to protect LDL parti-
cles 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, biliru-
bin is an important direct scavenger of short-
lived, highly active oxidants such as hydroxyl rad-
ical, peroxyxynitrite, or its scission products nitro-
gen dioxide and carbonate radical [89]. Rather,
bilirubin may be especially adept at "fixing" the
primary oxidant damage induced by these oxi-
dants 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 bil-
irubin to encounter these primary lesions before
they lead to permanent damage. Furthermore,
these primary lesions may be relatively weak oxi-
dants – compared to hydroxyl radical or nitrogen
dioxide, for example – so they would be more
likely to preferentially extract electrons from bil-
irubin's C-10 methylene, an eager electron donor.
Thus, biliverdin would be the chief product of bil-
irubin's antioxidant activity, enabling bilirubin to
be recycled.

473 But these considerations are still dodging an
474 important issue – how can intracellular free bilirubin,
475 in concentrations in the low nanomolar range,
476 compete effectively as a radical scavenger with
477 other intracellular antioxidants – including ascorbate,
478 urate, and reduced sulfhydryls – that are
479 present in millimolar or near-millimolar concentrations?
480 The efficacy of a scavenger is contingent on
481 the rate at which it can make contact with oxidants,
482 and that rate can be expected to be proportional to the
483 concentration of the scavenger. The intracellular
484 concentration of bilirubin, even after HO-1 induction,
485 can be expected to be over 10,000 times lower than
486 that of ascorbate, another versatile radical scavenger.
487 The fact that, as Snyder has emphasized, reversible
488 reduction by biliverdin reductase multiplies the
489 scavenging potential of the bilirubin pool, does not
490 alter the fact that the size of this pool intracellularly
491 is quite small compared to that of other effective
492 physiological scavengers. What makes bilirubin such
493 a remarkably effective antioxidant?

495 The likely explanation is that bilirubin's chief
496 role is not to act as a radical scavenger, but rather
497 as a potent and specific inhibitor of the membrane-bound
498 NADPH oxidase, a key source of oxidants not only in
499 phagocytes, but in a high proportion of non-phagocytic
500 cells as well [91]. Indeed, the results of a number of
501 studies can be interpreted as evidence that HO-1
502 induction and/or bilirubin suppress superoxide
503 production under circumstances in which the prime
504 source of superoxide is likely to be NADPH oxidase
505 [92–99,54,100,101]; direct scavenging of superoxide
506 by bilirubin is not a likely explanation for these
507 findings, since bilirubin's activity in this regard is
508 unremarkable – less than that of ascorbate [6,8,10],
509 which is present in much higher concentrations. Nonetheless,
510 the authors of these studies have usually attributed
511 bilirubin's potent antioxidant activity to its radical
512 scavenging activity. It is therefore gratifying to note
513 that, in a recent study, Boczkowski and colleagues
514 at last attribute bilirubin's antioxidant effect to
515 inhibition of the activation of NADPH oxidase [102].
516 These investigators have demonstrated that bilirubin
517 pre-treatment suppresses superoxide production by
518 macrophages stimulated by LPS, and likewise
519 suppresses superoxide production by aortic slices
520 obtained from rats injected with endotoxin; lucigenin-
521 dependent chemiluminescence was used in this study
522 to quantify superoxide production – a technique which
523 directly measures superoxide, rather than its
524 downstream oxidant products. The impact of bilirubin
525 was dose-dependent in the concentration range 1–
526 50 μM (most of this bilirubin was albumin-bound,

527 as fetal calf serum was included in the assay), and
528 thus was likely to be physiologically relevant. These
529 authors showed that bilirubin did not influence the
530 expression of the components of NADPH oxidase –
531 consistent with the fact that bilirubin's antioxidant
532 impact was rapid in onset. A previous, much older
533 study which Boczkowski cites purporting to demonstrate
534 that bilirubin inhibits assembly of NADPH oxidase
535 in leukocytes [103] may be of dubious relevance,
536 owing to the supraphysiologic concentrations of
537 bilirubin tested, the use of a cell-free assay, and
538 the fact that heme was more effective than bilirubin
539 in suppressing NADPH oxidase activation in this
540 study. However, very recently, two other research
541 groups have now confirmed that bilirubin does indeed
542 inhibit NADPH oxidase [104,105]; the study by Jiang
543 et al. extends our understanding by showing that
544 bilirubin can inhibit the membrane translocation of
545 p47, an essential step in activation of this enzyme
546 complex.

549 This new understanding of bilirubin's efficacy can
550 yield an elegantly simple explanation for the
551 antioxidant physiological role of HO-1: Excessive
552 intracellular oxidant activity triggers induction of
553 HO-1; the resulting intracellular generation of
554 bilirubin (via biliverdin and biliverdin reductase
555 activity) provides feedback suppression of NADPH
556 oxidase activity, one of the cell's chief sources
557 of oxidants. Moreover, the concurrently generated
558 CO may contribute at least modestly to this
559 inhibition of NADPH oxidase [106]. And this CO can
560 “pinch-hit” for the nitric oxide scavenged by
561 interaction with excess superoxide; like NO, CO
562 is an activator of guanylate cyclase. Finally,
563 diminution of the heme pool, in the longer term,
564 may suppress NADPH oxidase activity by decreasing
565 availability of its heme-dependent gp91^{phox} subunit
566 [97]. Thus, induction of HO-1 can work in multiple
567 complementary ways to provide feedback compensation
568 for the consequences of excessive NADPH activation.
569 The role of biliverdin reductase is to generate
570 bilirubin and to maintain it in its low-solubility
571 conformation in the face of oxidant assault.

573 Since NADPH oxidase plays a prominent role in the
574 anti-infective activity of white cells, it is reassuring
575 to note that clinicians have not reported any
576 evident increase in risk for infection in Gilbert
577 subjects; thus, moderate down-regulation of NADPH
578 oxidase activity may be compatible with adequate
579 immune protection. Nonetheless, Haga and colleagues,
580 having demonstrated that bilirubin can suppress
581 lymphocyte-mediated cytotoxicity, refer to “the
582 increased susceptibility to infection observed in
583 hyperbilirubinemic patients” [107,108]; other
584 relevant observations are noted below.

585 Strategies for harnessing Bilirubin's 586 health protective potential

587 How can the versatile antioxidant benefits of biliru-
588 bin be exploited in prevention and therapy? One
589 evident approach is to administer agents which
590 have HO-1 inductive activity in target tissues.
591 Phase II inducers, such as sulforaphane, lipoic acid,
592 green tea polyphenols, and various other bioavail-
593 able flavonoids have evident potential in this re-
594 gard. The intriguing report that alanine has HO-1
595 inductive activity [19] should be followed up, and
596 the extent to which the poorly understood vascu-
597 lar-protective activity of probucol [109–114] may
598 be mediated by HO-1 induction [20] should be
599 explored.

600 Biliverdin and phycobilins as antioxidant 601 nutraceuticals

602 Since bilirubin undergoes enterohepatic circulation
603 (free but not conjugated bilirubin is readily reab-
604 sorbed [115]), oral administration of bilirubin –
605 or, preferably, of its precursor biliverdin, which is
606 much more soluble and likely would be converted
607 to bilirubin in the intestinal mucosa - has the po-
608 tential to raise serum and tissue bilirubin levels.
609 A handful of studies have in fact evaluated the im-
610 pact of biliverdin supplementation in rodents. Back
611 in 1993, Nakagami and colleagues reported that
612 oral administration of biliverdin (5 mg/kg) an hour
613 before injection of Forssman antiserum suppressed
614 the resulting anaphylaxis and cut the death rate in
615 half; this effect was thought to stem from biliver-
616 din's ability to inhibit complement activation
617 [116]. Much more recently, Bach and colleagues
618 have reported that biliverdin, administered orally
619 at a dose of 50 $\mu\text{mol/kg}$ 2–3 times daily, greatly in-
620 creased cardiac allograft survival in mice; two-
621 thirds of the mice accepted the grafts and devel-
622 oped long-term tolerance to the transplanted
623 alloantigens, whereas all grafts in the control group
624 were rejected in a mean time of 11.5 days [117].
625 This effect was believed to reflect down-regulation
626 of NF-kappaB activation in T lymphocytes. Hope-
627 fully, studies will soon be forthcoming evaluating
628 the impact of oral biliverdin on vascular function.

629 Endogenous production of heme has been esti-
630 mated at 300–400 mg daily [118] – giving rise to
631 a nearly equivalent amount of bilirubin. Only a por-
632 tion of an orally administered biliverdin dose would
633 be absorbed, and first-pass hepatic metabolism
634 would clear some of this. Thus, it can be antici-
635 pated that supplemental daily intakes of 500 mg
636 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 biliver-
din at an affordable cost. Chemical synthesis of bil-
iverdin is presumably complex and costly. Thus,
developments in biotechnology or organic chemis-
try will be required before biliverdin supplementa-
tion 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 phyco-
cyanins, which function to "harvest" visible light
in chloroplasts. The three most prominent phycobi-
lins are phycocyanobilin (blue), phytochromobilin
(red), and phycoerythrobilin (red); they are syn-
thesized from biliverdin by reduction reactions
and isomerizations which produce a range of struc-
tures that absorb different portions of the visible
spectrum, and that are capable of being enzymati-
cally ligated to apoproteins. However, these
structural alterations are fairly trivial, affecting
only the ends of the molecules, such that the cen-
tral C-10 region remains unaltered and the sub-
stantial 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 demon-
strated that phycobilins are good substrates for bil-
iverdin 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 com-
pounds ("phycorubins") that are structural ana-
logs of bilirubin, and that seem likely to possess
somewhat comparable physiological activity. In-
deed, this may help to explain why oral administra-
tion of algae or of phycocyanin has shown
remarkably versatile and potent antioxidant, anti-
inflammatory, and anti-allergic activities in a num-
ber 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 induc-
tion; [102,132] inhibition of NADPH oxidase activa-
tion 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 replicat-
ing the range of health benefits conferred by

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693 elevated bilirubin levels. Indeed, Inoguchi has re- 749
694 cently observed that phycocyanobilin dose-depend- 750
695 ently inhibits the NADPH oxidase activity of 751
696 human cell cultures (endothelial, smooth muscle, 752
697 and mesangial) in the concentration range 1– 753
698 20 μM ; the inhibitory effects of biliverdin are quite 754
699 similar in these cell cultures (Toyoshi Inoguchi, 755
700 personal communication). 756

701 Phycocyanins constitute a high proportion of the 757
702 total protein content of many algae; [126] more- 758
703 over, when algae grown in the dark are fed delta- 759
704 aminolevulinic acid, they produce and secrete free 760
705 phycobilins [133]. Phycobilins can be extracted 761
706 from algae and cyanobacteria by prolonged treat- 762
707 ment with boiling methanol, which cleaves the thi- 763
708 oether linkage between phycobilins and their 764
709 apoproteins; a relatively selective extraction can 765
710 be achieved if algae are pre-extracted with cold 766
711 methanol, which will remove other chromophores 767
712 such as chlorophyll and carotenoids [134–136]. 768
713 *Spirulina* is one of the richest known natural 769
714 sources of phycocyanobilin, and is used commer- 770
715 cially for production of phycocyanin (employed as 771
716 a food dye and as a chromophore tag in biological 772
717 research) [137,138]. Unfortunately, since *Spirulina* 773
718 is obligately phototrophic, it is difficult to produce 774
719 efficiently in bulk; organisms that can be grown 775
720 heterotrophically might ultimately have greater 776
721 potential for commercial phycobilin production 777
722 [139,140]. 778

723 An additional possibility is that genetic engi- 779
724 neering could be employed to convert chloro- 780
725 phyll-rich plants into rich sources of biliverdin 781
726 and phytochromobilin. Although most of the pro- 782
727 toporphorin IX produced in green plants is used for 783
728 chlorophyll synthesis, a small portion of it is con- 784
729 verted to phytochromobilin via heme and biliver- 785
730 din. Phytochromobilin is the chromophore for 786
731 phytochrome proteins which act as light detectors 787
732 that regulate plant development (rather than har- 788
733 vesting light for biosynthesis, as in algae) [141]. 789
734 Insertion of $\text{Mg}(2+)$ into protoporphorin IX, cata- 790
735 lyzed by Mg-chelatase, commits it to chlorophyll 791
736 synthesis, whereas insertion of $\text{Fe}(2+)$ via the en- 792
737 zyme ferrochelatase generates heme [142]. Mg- 793
738 chelatase is at a marked competitive advantage 794
739 in this regard, in as much as it is K_m for protopor- 795
740 phyrin – 13 nM – is orders of magnitude lower than 796
741 that of plant ferrochelatase, determined to be 797
742 2.4 μM in peas [143]. In contrast, the ferrochela- 798
743 tase of non-photosynthetic organisms appears to 799
744 have a much higher affinity for protoporphyrin – 800
745 in *Saccharomyces*, K_m is 50 nM [144]. In green 801
746 plants that synthesize large quantities of chloro-
747 phyll, it might prove feasible to divert a much high-
748 er proportion of protoporphyrin IX to heme and

biliverdin by transfecting them with a constitu-
tively active yeast ferrochelatase, modified to pro-
mote 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 muta-
tion might also promote this goal. In this way,
green plants might be turned into rich sources of
biliverdin and phytochromobilin. Since the chloro-
phyll content of spinach is greater than 1% of dry
weight, a spinach which made comparable amounts
of biliverdin/phytochromobilin could be of consid-
erable utility.

A potential snag is that increased heme levels
feed back to suppress synthesis of delta-aminolev-
ulinic 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. Alterna-
tively, a truncated form of glutamyl-tRNA reduc-
tase (rate-limiting for ALA synthesis in plants) not
susceptible to inhibition by heme been character-
ized in barley; [146] transfection of this would pre-
sumably 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]. Presum-
ably, heme synthesis (and thus phycobilin sythesis)
in this organism could be maximized by feeding it
ALA while insuring that expression of heme oxygen-
ase 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 hyperbili-
rubinemia associated with Gilbert syndrome re-
flects underactivity of hepatic UGT1A1, it may
be feasible to induce an “iatrogenic Gilbert syn-

drome" 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 2.51 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 adjuvant 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 levels, clinicians have evidently observed raised bilirubin during probenecid therapy, as such therapy is listed as a possible cause of hyperbilirubinemia in the differential diagnosis of this syndrome [155]. Thus, it would be of great interest to formally study the dose-dependent impact of probenecid 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, in 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 [190,165,191], 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-

911 bation of chondrocyte function that leads to carti- 967
912 lage degradation in arthritis [206–210]. This en- 968
913 zyme complex may also play a pathogenic role in 969
914 fibrotic disorders, inasmuch as both the production 970
915 of TGF-beta and the responsiveness of collagen 971
916 synthesis to this cytokine are up-regulated by 972
917 NADPH oxidase-derived oxidants; [211–213] acti- 973
918 vation of NADPH oxidase appears to play a key 974
919 role in TGF-beta signaling [214,215]. Thus, it is 975
920 not surprising that HO-1 has been reported to exert 976
921 bilirubin-mediated antifibrogenic effects in myofi- 977
922 broblasts [43], that bilirubin administration as well 978
923 as overexpression of HO-1 ameliorate bleomycin- 979
924 induced pulmonary fibrosis in rats [216–218], and 980
925 that a case of idiopathic pulmonary fibrosis was 981
926 noted to remit following onset of hyperbilirubine- 982
927 mia [219]. Furthermore, chronic activation of 983
928 NADPH oxidase in the fibroblasts of scleroderma 984
929 patients has been reported to sustain the excess 985
930 proliferation and collagen production of these cells 986
931 [220]. On the other hand, activation of NADPH oxi- 987
932 dase can also induce excessive degradation of 988
933 ground substance. Cigarette smoke exposure pro- 989
934 motes emphysema by inducing expression of elas- 990
935 tase in bronchial epithelium; activation of NADPH 991
936 oxidase is essential to this induction [221]. 992

937 Osteoclasts express NADPH oxidase activity, and 993
938 the hydrogen peroxide that evolves from such 994
939 activity plays a role in osteoclast differentiation, 995
940 while also promoting osteoclast-mediated bone 996
941 resorption [222–226]. Conversely, genetically- 997
942 linked osteopetrosis, in both humans and mice, is 998
943 associated with a deficit in NADPH oxidase activity 999
944 [222,227]. The accelerated bone resorption associ- 1000
945 ated with estrogen deficiency may reflect down- 1001
946 regulation of antioxidant mechanisms that coun- 1002
947 teract the impact of hydrogen peroxide on osteo- 1003
948 clasts [228,229]. Thus, pharmaceutical inhibition 1004
949 of NADPH may have potential for prevention of 1005
950 postmenopausal osteoporosis. Inasmuch as Rac is 1006
951 essential for optimal NADPH oxidase activity in 1007
952 osteoclasts [230]. it is tempting to speculate that 1008
953 a decrease in the geranylgeranylation of Rac medi- 1009
954 ates, at least in part, the favorable impact of stat- 1010
955 ins and nitrogen-containing bisphosphonates on 1011
956 bone density [231–236]. 1012

957 Increased NADPH oxidase activity is a mediator 1013
958 of increased mitogenic activity and/or cell survival 1014
959 in various types of cancer; [237–246] moreover, 1015
960 activation of NADPH oxidase plays an obligate role 1016
961 in the process of angiogenesis [247,248]. The 1017
962 NADPH oxidase activity of phagocytes undoubtedly 1018
963 contributes to the increased risk for mutagenesis 1019
964 and cancer associated with chronic inflammation 1020
965 [249–251]. This enzyme is activated in microglia 1021
966 in Alzheimer's disease [252,253], and appears to 1022

mediate the pro-apoptotic impact of amyloid-beta 967
peptide on neurons in this disorder [254]. A possi- 968
ble role for microglial NADPH oxidase activity in 969
the genesis of Parkinson's disease has also been 970
suggested [255,256]. 971

In obese diabetic KKAY mice, activation of 972
NADPH oxidase in adipocytes has been shown to 973
be a key mediator of the "inflamed" adipocyte 974
phenotype, associated with increased production 975
of TNF- α and other inflammatory cytokines, adipo- 976
cyte insulin resistance, and the metabolic syn- 977
drome. Treatment of such mice with the NADPH 978
oxidase inhibitor apocynin ameliorated the in- 979
flamed state of their white adipose tissue, while 980
reducing elevated serum levels of glucose, triglyc- 981
erides, and insulin [257]. However, Goldstein and 982
colleagues have shown that, in some tissues, 983
including adipocytes, insulin-mediated activation 984
of NADPH plays a role in amplifying insulin signal- 985
ing, as the derived hydrogen peroxide inhibits 986
PTP-1B, a phosphotyrosine phosphatase that tar- 987
gets the insulin receptor; [258,259] this may be 988
a specific incidence of the more general phenom- 989
enon of NADPH oxidase activation up-regulating 990
growth factor signaling. There do not appear to 991
be any studies examining the impact of NADPH 992
oxidase inhibition on insulin function in lean ani- 993
mals. To the extent that failure to inactivate 994
PTP-1B acts as a countervailing negative factor 995
when NADPH oxidase inhibition is employed in 996
insulin resistant or diabetic patients, this effect 997
might be compensated by concurrent administra- 998
tion of cinnamon extract, the insulin-sensitizing 999
impact of which is thought to reflect inhibition 1000
of PTP-1B [260,261]. 1001

Activation of microglia is a common feature of 1002
a number of chronic neurodegenerative brain dis- 1003
orders. The superoxide produced via NADPH oxi- 1004
dase in these microglia yields peroxynitrite upon 1005
condensing with nitric oxide generated from iNOS 1006
that likewise is induced in these disorders; this 1007
peroxynitrite is suspected to mediate much of 1008
the neuronal death and dysfunction associated 1009
with these syndromes [262]. Moreover, recent evi- 1010
dence suggests that activation of NADPH oxidase 1011
within neurons may be a key mediator of excito- 1012
toxic neuronal death [263] – a phenomenon that 1013
contributes to neuron loss in stroke and neurode- 1014
generative disorders [264,265]. Thus, NADPH oxi- 1015
dase inhibition may have a role to play in the 1016
prevention and treatment of various neurodegen- 1017
erative conditions, possibly including common syn- 1018
dromes such as Alzheimer's and Parkinson's 1019
diseases [266,252,253,255,267]. 1020

Superoxide is emerging as a key mediator of 1021
hyperalgesia associated with various chronic pain 1022

1023 syndromes; thus, cell permeable superoxide dismu- 1079
1024 tase mimetics have an ameliorative impact on such 1080
1025 syndromes [268–271]. Neuronal NADPH oxidase 1081
1026 seems likely to be a prominent source of the super- 1082
1027 oxide involved in hyperalgesia [272,273]. Indeed, 1083
1028 the hyperalgesic impact of nerve growth factor 1084
1029 has been traced to NADPH oxidase-generated 1085
1030 superoxide, which via p38 MAP kinase activation 1086
1031 promotes translation of TRPV1, an ion channel 1087
1032 receptor which is a key mediator of nociception 1088
1033 [274–276]. Superoxide can also promote activation 1089
1034 of c-Src kinase, which boosts TRPV1 activity 1090
1035 [277,274]. And, since NO, acting via cGMP, sup- 1091
1036 presses hyperalgesia [278,279], superoxide's 1092
1037 antagonism of NO bioactivity would be expected 1093
1038 to boost pain reception. 1094

1039 Exposure of keratinocytes to UVB triggers activa- 1095
1040 tion of NADPH oxidase, which appears to be the 1096
1041 chief source of the oxidative stress involved in 1097
1042 UVB-mediated skin damage [280,281]. Thus, inhibi- 1098
1043 tion of NADPH oxidase may have potential for pre- 1099
1044 ventation both of the acute effects of UVB exposure 1100
1045 as well as longer term consequences of chronic 1101
1046 exposure such as cosmetic skin aging. 1102

1047 Activation of NADPH oxidase appears to play an 1103
1048 essential role in the induction of inducible NO syn- 1104
1049 thase triggered by bacterial lipopolysaccharide 1105
1050 [282,283]. This may rationalize observations that 1106
1051 pre-treatment with hemoglobin (which induces 1107
1052 HO-1), bilirubin, or oral phycocyanin prevents cir- 1108
1053 culatory collapse and lethality in rodents subse- 1109
1054 quently infused with lipopolysaccharide [284– 1110
1055 286,132]. Thus, bilirubin and its homologs may 1111
1056 have potential for controlling septic shock – an ef- 1112
1057 fect recently demonstrated with in bilirubin-in- 1113
1058 fused rats [102]. 1114

1059 In disorders in which phagocyte hyperactivity 1115
1060 plays a pathogenic role, such as emphysema, gas- 1116
1061 trointestinal ulceration, acute respiratory distress 1117
1062 syndrome, and rheumatoid arthritis, partial sup- 1118
1063 pression of phagocytic NADPH oxidase activity 1119
1064 may be of benefit [287–289]. Clearly, however, 1120
1065 excessive or untimely inhibition of this enzyme 1121
1066 could increase the risk for, or severity of, infec- 1122
1067 tions; not surprisingly, bilirubin has been shown 1123
1068 to impair the bactericidal activity of neutrophils 1124
1069 in vitro [290]. However, this effect only became 1125
1070 statistically significant at an albumin-bound bilirubin 1126
1071 concentration of 150 μM – about fivefold the 1127
1072 concentration seen in Gilbert syndrome; thus, 1128
1073 moderate elevations of bilirubin may not be harm- 1129
1074 ful in this regard. NADPH oxidase activity plays a 1130
1075 role in the expression of MHC class II by antigen- 1131
1076 presenting cells [291], rationalizing a recent report 1132
1077 that bilirubin inhibits MHC class II expression by 1133
1078 endothelial cells [292]. Thus, inhibition of NADPH 1134

oxidase activity may influence antigen-specific im-
mune defenses; indeed, biliverdin administration is
reported to promote survival of cardiac allografts
in rats [117].

Activation of NADPH oxidase may play a promi-
nent role in HIV-1 infection – boosting the tran-
scription of HIV proteins, while also mediating
some of the pathogenic consequences of HIV infec-
tion. Several HIV proteins – Tat, Nef, and gp120 –
can stimulate NADPH oxidase activity [293–299].
Since oxidants can up-regulate NF-kappaB activa-
tion [300,301], and this transcription factor pro-
motes the transcription of HIV proteins via NF-
kappaB response elements in the long terminal re-
peat (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 sup-
presses 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 cera-
mide-mediated apoptosis; this generation of cera-
mide, 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-re-
lated dementia. Peroxynitrite production by HIV-
infected microglia might also contribute in this re-
gard [307] – a phenomenon that likewise is
dependent on NADPH oxidase activation. With re-
spect to the possibility of targeting NADPH oxi-
dase in the management of HIV infection,
however, it should be noted that the immunosup-
pressive potential of such a strategy might make
its use problematical in patients who have pro-
gressed 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 addi-
tion, activation mechanisms for NADPH oxidase
may vary from tissue to tissue. Thus, it is quite con-
ceivable 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 NAPDH 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 peroxyxynitrite derived from this enzyme complex induce reversible oxidation of sulfhydryl 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 feedback mechanism by raising systemic levels of bilirubin or its homologs.

Summing up

Accumulating evidence provides strong support for the thesis that bilirubin, owing to its versatile and potent antioxidant activity, and the ability of biliverdin reductase to rapidly recycle it, makes a crucially important contribution to the body's antioxidant defenses. And, unlike antioxidant enzymes which may be of comparable importance in this regard, its tissue concentrations may be highly susceptible to modulation with practical, well tolerated strategies. Further rodent and clinical research is needed to better define measures that can achieve marked increases in serum and tissue bilirubin comparable to those observed in Gilbert syndrome, associated with marked protection from coronary disease and cancer in current epidemiology. Heme oxygenase inducers, biliverdin supplementation, and agents which slow hepatic conjugation of bilirubin, should be evaluated in this regard. Phycobilin supplementation may have the potential to replicate the antioxidant benefits

conferred by elevated bilirubin. Although it will be difficult to assess the long-term health impacts of such strategies, shorter term clinical studies focusing on endothelial function, DNA stability (e.g. comet assay, 8-hydroxy-2'-deoxyguanosine levels), and markers of inflammation could likely provide insight into the true health protective potential of such measures.

In light of the key role which oxidants play as signaling mediators and bactericidal agents in immune cells, it might prove advisable to discontinue bilirubin-elevating measures (or phycobilin supplementation) temporarily in patients suffering from severe or persistent infections. Ultimately, the profound antioxidant activity of bilirubin may help us to gain a clearer understanding of why Nature has developed and conserved mechanisms for oxidant generation.

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