Bilirubin, a curse and a boon

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Unconjugated bilirubin is a curse at high concentrations, producing apoptosis and cell death, but a boon at more physiological levels, protecting cells against oxidant damage

Both hydrophobic bile salts^{1,2} and unconjugated bilirubin (UCB)³ induce apoptosis in cultured cells at moderately elevated concentrations and cell necrosis at higher concentrations. Retention of bile salts in cholestasis is believed to cause secondary damage to hepatocytes,⁴ and retention of UCB in severe neonatal jaundice is known to cause bilirubin encephalopathy.⁵ For both agents, the cytotoxicity results from damage to mitochondrial membranes, with collapse of the transmembrane potential and generation of a mitochondrial membrane permeability transition,^{1,2,6} and ursodeoxycholic acid or its amidates can prevent apoptosis by inhibiting this process.^{1,2,7} It is therefore surprising that, as reported by Granato *et al* in this issue of *Gut*,⁸ UCB, at concentrations far above those known to be cytotoxic to neurones and astrocytes,³ is protective against apoptosis induced in cultured rat hepatocytes by the hydrophobic bile salt glycochenodeoxycholate (GCDC) [see page 1774]. This finding raises interesting questions concerning whether UCB is a curse or a boon, and why various organs and tissues have such different susceptibilities to toxicity from these substances.

EXPERIMENTAL CONSIDERATIONS

Like many previous *in vitro* studies of the cytotoxicity of UCB⁹ and bile salts,¹⁰ the studies of Granato and colleagues⁸ were performed at concentrations of the unbound fraction far above those that are clinically relevant. Although the incubation media contained 18 μ M bovine serum albumin, concentrations of UCB and GCDC greatly exceeded the high affinity binding capacity of 1 mol/mol albumin. The final unbound concentrations of UCB (B_f) were also far above aqueous saturation,¹¹ so the pigment must have been heavily self aggregated.^{12,13} Unbound concentrations of GCDC were also well above those seen in plasma in cholestasis, questioning the relevance of the results to clinical conditions.¹⁰ Furthermore, although UCB was recrystallised, polar

derivatives had not been extracted with alkali,¹⁴ and surface active impurities, which may have contributed to the toxicity, were not removed from GCDC by available methods.^{15,16}

IS BILIRUBIN A CURSE OR A BOON?

Bilirubins were once regarded as toxic wastes, of importance only when excessively high levels of UCB led to the development of encephalopathy.⁵ It is now appreciated that bilirubins and biliverdin are extremely potent antioxidants,^{17,18} and that these antioxidant properties may be cytoprotective,^{19,20} even at physiological concentrations. A new finding of the Granato paper⁸ was that the synthetic ditaurate conjugate of bilirubin, a satisfactory surrogate for the natural glucuronosyl conjugates, was nearly as potent as UCB in protecting hepatocytes against toxicity from GCDC.

The antioxidant effects of bilirubins and biliverdin are mediated largely by sacrificial oxidation of these pigments,²¹ consuming reactive oxygen species that could damage membrane lipids. However, UCB also regenerates other natural antioxidants,¹⁸ and traps a variety of reactive oxygen species.²² Binding to plasma albumin,²² and possibly to cytosolic proteins,²³ enhances the antioxidant potency of UCB. Thus both bound and unbound UCB and conjugated bilirubins in plasma and the cytosol are available to protect cell membranes from lipid peroxidation. By contrast, it is only "free" UCB (B_f), the <0.1% of total plasma UCB that is not protein bound,^{24,25} that can enter cells by passive or facilitated diffusion²⁶ that determines its toxic effects intracellularly.²⁴

A recent meta-analysis of *in vitro* studies conducted at relevant B_f levels revealed that cultured neurones and astrocytes suffer both plasma membrane and mitochondrial damage at B_f levels slightly to modestly above the 70 nM aqueous solubility limit of UCB;³ this suggests that the inimical species might be soluble oligomers and/or small colloidal aggregates of UCB. By contrast, exposure to UCB at B_f levels below

70 nM protects cultured neurones and their mitochondria against oxidative stress induced by hydrogen peroxide; this protection is diminished at higher B_f values where the toxic effects of UCB became dominant.²⁰

Low levels of endogenous UCB, generated intracellularly by microsomal haeme oxygenases, likewise protect against oxidative damage to cells.^{19,27-30} This raises a seeming paradox—how can such low (nanomolar) concentrations of UCB be protective against the much higher (micromolar) concentrations of reactive oxygen species, especially as UCB is destroyed in the process? Several mechanisms may be involved: (1) cyclical regeneration of UCB from biliverdin by the huge excess of cytosolic biliverdin reductase³¹; (2) upregulation of haeme oxygenase 1 activity and production of biliverdin (and ultimately UCB) by many stimuli, including oxidative stress²⁷; and (3) replenishment of intracellular UCB via uptake after dissociation from the huge reservoir of UCB bound to plasma albumin.

DIFFERENTIAL SUSCEPTIBILITIES TO BILIRUBIN TOXICITY

Granato and colleagues⁸ reported that rat hepatocytes were unaffected by B_f levels approximately three orders of magnitude higher than those that cause cell damage in rodent astrocytes and neurones,³ confirming *in vivo* observations in jaundiced Gunn rats that oxidative phosphorylation and respiration in the liver is unimpaired even in animals with bilirubin encephalopathy and kernicterus.³² Apparently, the resistance of the liver to UCB toxicity allows that organ to benefit from the antioxidant properties of the pigment, even at very high UCB levels. In the CNS, regional differences in sensitivity to UCB toxicity are manifested by selective accumulation of UCB only in certain areas of the brain in kernicteric neonates and Gunn rats.³ What might account for these differences?

Cells might be protected against accumulation of UCB by transporters that export UCB from the cell back into the blood and by conjugation and/or oxidation of UCB to non-toxic polar derivatives.³³ When the cellular export and metabolism of UCB are insufficient to keep its total intracellular concentration low, cells might be protected by intracellular proteins that bind UCB,⁵ or by changes in mitochondrial membrane composition.³⁴

Transporters

Both hepatocytes and a variety of CNS cells express two classes of ATP binding cassette (ABC) transporters, multidrug resistance P-glycoproteins (MDR/PGPs)

and the multidrug resistance associated proteins (MRPs) that can protect cells against accumulation of toxic substances by exporting them back into the plasma.³⁵ For unconjugated organic anions, the most important of such exporters are MDR1/mdr1, MRP1/Mrp1, and MRP3/Mrp3. In the hepatocyte, MDR1 is localised apically, and MRP1 and 3 basolaterally.³⁵ In the cholestatic liver, or after infusion of bile acids, the two Mrps are upregulated to limit accumulation of organic anions into the hepatocyte.³⁶ Persistent hyperbilirubinaemia likewise enhances the conjugation and excretion of bilirubin³⁷ because binding of UCB to the constitutive androstane receptor upregulates expression of ligandin, UGT1A1, and MRP2.³⁸ It is not known if Mdr1a/b or Mrp 1 and 3 are induced by UCB.

ABC transporters in the capillary endothelium (blood-brain barrier) and/or the choroid plexus (CP) epithelium (blood-cerebrospinal fluid (CSF) barrier) have been shown to play an important role in limiting the accumulation of organic anions and other small molecules in the CNS and CSF, respectively.^{33–39} As yet however there is only weak evidence that UCB is carried by any ABC transporters. Although some data have suggested that Mdr1a⁴⁰ and MDR1⁴¹ limit the accumulation of UCB in CNS cells, these results are inconclusive due to the use of vastly excessive UCB concentrations.⁹ UCB is reportedly a weak substrate for MDR1,⁴² and a role for MRP1 in UCB transport has been proposed,^{43–44} but not directly proven. Even if they do transport UCB, the presence of Mrp1 and Mdr1 in the blood-brain barrier and CP cannot explain the preferential accumulation of UCB in only selected areas of the kernicteric brain.⁵

Conjugation

Hepatocytes are the major site of bilirubin conjugation, mediated by the specific microsomal UDPGA-bilirubin glucuronosyl transferase, UGT1A1.⁴⁵ In the brain, CP epithelial cells express glucuronosyl transferases for a variety of other organic anions³³ but UGT1A1 has yet to be demonstrated either here or in astrocytes or neurones. Even if present, UGT1A1 in the CP could play no role in protecting cultured astrocytes or neurones from UCB toxicity in vitro. In the paper by Granato and colleagues,⁸ bilirubin conjugation by their cultured hepatocytes was extremely low and thus unlikely to account for the resistance of these cells to UCB toxicity.

Oxidation

In the liver, UCB can be oxidised by the microsomal mixed function

monooxygenases, CYP1A1 and CYP1A2, but basal expression is low,⁴⁶ although readily inducible by indole-3-carbinol and aryl hydrocarbons.^{21–47} In many regions of the brain, low constitutive levels of CYP1A2 are found in neurones and of CYP1A1 in the capillary endothelium of the CP and arachnoid.³³ CYP1A1 in liver is transcriptionally induced by elevated UCB levels in jj Gunn rat pups,⁴⁶ mediated by binding of UCB or an oxidation product to the aryl hydrocarbon receptor.⁴⁸ In the latter paper, significant upregulation of CYP1A1 occurred after exposure of hepatoma cells to UCB under conditions similar to those used by Granato and colleagues,⁸ suggesting that increased oxidation of UCB contributed to the resistance of hepatocytes to UCB toxicity.

Cytosolic binding proteins

In the hepatocyte, tight binding to two different cytosolic glutathione S-transferases, Y protein (ligandin) and Z protein, limits the free cytosolic concentrations of UCB⁴⁹ and bile salts,^{50–51} respectively. Ligandin is little expressed in the CNS,⁵² suggesting that the abundance of ligandin in hepatocytes may be a key factor in their greater resistance to UCB toxicity compared with neurones and astrocytes. The reputed antioxidant potency of UCB bound to cytosolic binding proteins²³ could account for the protective effect of UCB against GDC mediated apoptosis in hepatocytes,⁸ which is mediated by generation of reactive oxygen species.⁵³

Mitochondria from rat livers are themselves more resistant than mitochondria from rat brain to impairment of respiration and oxidative phosphorylation by UCB concentrations in the micromolar range,^{54–55} even though the net uptake of ¹⁴C-UCB is similar in both preparations.⁵⁵ These findings suggest that intrinsic changes in the mitochondrial membrane contribute to the greater resistance of hepatocytes to apoptosis caused by hydrophobic bile salts. Additionally, hepatocytes and liver mitochondria from bile duct ligated rats are more resistant to the mitochondrial permeability transition than are the specimens from sham operated controls, and this may be mediated by an increase in mitochondrial cardiolipin content.³⁴

CONCLUSIONS

UCB is a curse at high concentrations, producing apoptosis and cell death, but a boon at more physiological levels, protecting cells against oxidant damage. Differences in organ or cell susceptibility to bilirubin toxicity may be related to differences in expression of ABC transporters that can export UCB, conjugation and oxidation of the pigment,

binding of the pigment to cytosolic binding proteins, and intrinsic differences in the composition of mitochondrial membranes. Conjugation and oxidation convert the pigment to polar non-toxic derivatives which themselves may be antioxidants and/or regulate the metabolism and transport of bilirubin and bile salts. Binding of UCB to ligandin decreases the concentration of the toxic unbound fraction and may enhance the antioxidant potency of the bound fraction. Much additional work, preferably performed with UCB at relevant concentrations, is needed to verify or disprove these possibilities.

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