Bilirubin, Copper-Porphyrins, and the Bronze-Baby Syndrome

Antony F. McDonagh, PhD

Controlled in vitro spectroscopic measurements reveal that bilirubin does not photosensitize the degradation of copper-porphyrins, as has been proposed for the mechanism of the bronze-baby syndrome, an uncommon side-effect of phototherapy. Calculations also show that copper-porphyrins are unlikely to cause the "bronzing." In conclusion, the copper-porphyrin hypothesis is photochemically implausible. *(J Pediatr 2011;158:160-4)*

The bronze-baby syndrome (BBS) is an uncommon side-effect of phototherapy in which the patient's skin pigmentation darkens.[1-3] Although potentially alarming, the syndrome is harmless, and pigmentation returns slowly to normal if phototherapy is discontinued. It is apparent that the syndrome occurs only in newborns with cholestasis and with elevated plasma levels of both unconjugated and conjugated bilirubin. Not all babies with cholestasis develop the BBS during phototherapy, but all babies who do should be investigated for underlying liver disease.

Several explanations for the BBS have been proposed. One that has gained credence is that it is caused by deposition in the skin of uncharacterized copper-porphyrin derivatives, whose formation is photosensitized by bilirubin.[4-7] Evidence for their formation is a reported slight increase in absorbance near 520 to 600 nm of serum from a bronze baby when irradiated with tungsten light in vitro and similar increases when solutions of copper(II)-protoporphyrin (CuP) in cord blood serum or in solutions of human serum albumin (HSA) were irradiated in vitro with visible light in the presence of bilirubin.[7] This increased absorbance was attributed to CuP derivatives formed in a photochemical reaction between bilirubin and copper-porphyrins in the blood. Their accumulation during phototherapy was proposed as the cause of the hyperpigmentation in the BBS. Despite the tenuous supporting evidence, the copper-porphyrin theory has gained acceptance in the literature.[8,9] However, copper-porphyrins are chemically robust molecules. Moreover, bilirubin is a poor photosensitizer that readily loses photoexcitation energy by isomerization and other radiationless processes.[10] Those properties are inconsistent with the copper-porphyrin hypothesis. This inconsistency led me to reexamine spectroscopically the proposed photochemical interactions between bilirubin and CuP.

Methods

Cu(II)-protoporphyrin (CuP) was a generous gift from Dr. Jerry Bommer, Frontier Scientific (Logan, Utah). Bilirubin IXα was purified by preparative high-pressure liquid chromatography (HPLC) of a mixture of the IIIα, IXα, and XIIIα isomers[11] and contained <1% of IIIα and XIIIα isomers. Phosphate buffer (0.1 mol/L, pH 7.4), human serum albumin (HSA) (Fraction V), di-*n*-octylamine (for HPLC) and other chemicals were from Sigma-Aldrich Corp. (St. Louis, Missouri), except for MeOH, which was Fisher HPLC grade. Absorbance measurements were run on an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Santa Clara, California), and HPLC analyses were run as previously described.[12] Irradiations were run in stoppered quartz 1-cm optical-pathlength dual-compartment cells with an aperture in the top of the divider separating the two 0.5-cm pathlength compartments, permitting mixing of solutions in the two compartments by inversion. A 20 W Westinghouse Special Blue fluorescent tube (F20T12/BB; Westinghouse, Cranberry, Pennsylvania) filtered through a 4.5-mm–thick Plexiglass filter was used for irradiations. A narrow-band light source with maximum emission close to the BR absorption band was used rather than a broad-spectrum tungsten light source, as used by Rubaltelli et al,[7] to favor photoexcitation of bilirubin over CuP. The total distance from the light to the cuvette was 1 cm. Pigment solutions were prepared and manipulated by use of all-glass apparatus in a dark-room under orange safelights, and microbalances were used for all weighings. NaOH (0.1 mol/L), used for dissolving pigments, was made up fresh and purged with argon before use.

HSA stock solution. HSA stock solution. HSA (0.307 g) was dissolved in pH 7.4 buffer 50 mol/L.

Bilirubin/HSA and CuP/HSA solutions. Bilirubin (0.35 mg) or CuP (0.188 mg) was dissolved in 0.5 mL 0.1 mol/L NaOH, and the solution was transferred quantitatively into ~8 mL HSA stock solution. To this was added 0.5 mL 0.1 mol/L HCl and the total volume of the solution made up to exactly 10 mL in a volumetric flask with HSA stock solution to give final pigment concentrations of 59 μmol/L (bilirubin) or 26 μmol/L (CuP) in 87 μmol/L HSA solution.

BBS	Bronze-baby syndrome
CuP	Copper(II)-protoporphyrin IX
HPLC	High pressure liquid chromatography
HSA	Human serum albumin

From the Division of Gastroenterology and the Liver Center, University of California San Francisco, San Francisco, CA

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Photochemical Experiments

Irradiation of bilirubin/HSA and CuP/HSA separately. Bilirubin/HSA or CuP/HSA solutions were placed in the front compartment of a dual-compartment cuvette with HSA stock solution in the rear compartment. The cuvette was exposed to the light source and spectra were run before irradiation and at 5, 10, 20, 40, and 60 minutes after irradiation versus a blank with HSA solution in both cell compartments. After 60 minutes irradiation the Bilirubin/HSA and CuP/HSA solutions were sampled for HPLC.

Controls 1 and 2. For Control 1 exactly 1 mL of bilirubin/HSA was placed in the front compartment of the dual compartment cell and exactly 1 mL of CuP/HSA in the rear compartment. The cuvette was irradiated and spectra measured at intervals as above. After 60 minutes the contents of the two compartments were mixed, and a final spectrum was taken, as well as a sample for HPLC. Control 2 was run in exactly the same way but with bilirubin/HSA in the rear compartment and CuP/HSA in the front compartment. Two such control experiments were run to detect possible "inner filter" effects that might have influenced the experimental results.

Experiment. Exactly 1 mL bilirubin/HSA was placed in one compartment of the dual compartment cell and exactly 1 mL CuP/HSA in the other compartment. A spectrum was run, and then the contents of the two compartments were mixed by inversion. After another (t = 0 min) spectrum had been taken, the cuvette was irradiated, and spectra were taken at 5, 10, 20, 40, and 60 minutes after irradiation. The final solution was sampled for HPLC. Although only single experiments are shown, replicate experiments gave the same results.

Results

Irradiation of bilirubin in the presence of a molar excess of HSA led to a decrease in the intensity of the main visible absorption band, a shift in its absorption maximum to shorter wavelength (hypsochromic shift), and increased absorbance from ~520 to 650 nm (Figure 1, A). The overall loss of absorbance indicates conversion of bilirubin to colorless products, and the hypsochromic shift and increased absorbance at longer wavelengths indicate the formation of other products with visible absorbance. HPLC of the irradiated solution (Figure 1, C) after 60 minutes showed that much of the starting natural 4*Z*,15*Z* isomer of bilirubin had disappeared with conversion of part of this to faster eluting photoisomers (*E*-lumirubin, *Z*-lumirubin, and 4*Z*,15*E* bilirubin), which were identified by their UV-Vis absorbance spectra and comparison with authentic samples.[13,14] In contrast, under the same conditions the absorbance spectrum (Figure 1, B) of CuP showed only a very slight decrease in the Soret absorbance peak at 410 nm, with negligible change in the wavelength region above 450 nm. Thus CuP is stable under irradiation conditions that cause extensive photochemical isomerization and destruction of bilirubin.

To determine whether bilirubin photosensitizes degradation of CuP, as suggested,[5-7] an HSA solution containing both pigments was irradiated, and changes in the absorbance spectra were compared with those in control irradiations in which identical amounts of the two pigments were physically separated. Figure 2, A and B, shows the spectral changes observed on irradiation when the bilirubin/HSA and CuP/HSA solutions were physically separated in the dual compartment cuvette, in the one case with the bilirubin/HSA solution in the front compartment and in the other case with the bilirubin/HSA solution in the rear compartment. Figure 2, C, shows the spectral changes when identical volumes of bilirubin/HSA and CuP/HSA solutions were placed in the dual-compartment cell, but mixed before beginning the irradiation. The spectra before irradiation (which reflect the sum of the bilirubin/HSA and CuP/HSA spectra) and the spectral changes on irradiation (Figure 2, A, B, and C) for all 3 conditions were identical within the experimental error. In each case there was loss and a hypsochromic shift of the main bilirubin absorption band and increased absorbance from 520 to 650 nm. The results show that CuP and bilirubin do not interact in the presence of HSA, that neither one displaces the other from HSA, and, importantly, that bilirubin does not photosensitize the conversion of CuP to photoproducts. Furthermore, the identical rate of loss of bilirubin absorbance in the two controls and the experimental solution shows that CuP does not inhibit the photochemistry of bilirubin either by physical absorption or by quenching of excited states. This is further borne out by the HPLC chromatograms in Figure 2, H, which show that the array of bilirubin photoproducts after 60 minutes is not influenced by the presence of CuP in the same solution as bilirubin. Figure 2, D, E, and F, which are scale-expansions of the long-wavelength segments of Figure 2, A, B, and C, show that the increased absorbance above 520 nm, which was assumed by Jori et al[5,6] to reflect formation of CuP photoproducts, is seen in both control reactions in which the bilirubin/HSA and CuP/HSA solutions were physically separated and is identical in magnitude to that seen when the two pigments were present in the same solution. At the end of each control study the separated solutions in the two compartments of the cuvette were mixed and a final spectrum taken. Figure 2, G, compares these spectra with that of the final solution when the bilirubin/HSA and CuP/HSA solutions were mixed before irradiation. Within the experimental error they are identical. Finally, HPLC chromatograms of the final solutions after 60-minute irradiation (Figure 2, H) showed identical amounts of isomeric bilirubin pigments.

Discussion

The basic hypothesis of the Cu-porphyrin theory of the BBS is that in serum "Cu^{2+}-porphyrins undergo photodestruction sensitized probably by bilirubin yielding products with generalized absorption in the near-UV and red spectral regions; thus the brown discoloration."[7] This notion evolved