0031-3998/90/2701-0022$02.00/0
PEDIATRIC RESEARCH

Vol. 27, No. 1, 1990
Printed in U.S.A.

Bronze Baby Syndrome: An Animal Model

GIULIO JORI, ELENA REDDI, AND FIRMINO F. RUBALTELLI

Department of Pediatrics, University of Padova, and Department of Biology and C.N.R. Center for the Physiology and Biochemistry of Hemocyanins and Other Metallo-Proteins, University of Padova, Padova, Italy

ABSTRACT. We evaluated the appropriateness of an animal model for the bronze baby syndrome. Ligation of the common bile duct in adult Wistar rats induces an accumulation of porphyrins and copper in the liver and a 20% conversion of protoporphyrin IX into Cu(II)-protoporphyrin IX. Upon irradiation of these animals with superblue lamps, the plasma content of Cu(II)-protoporphyrin increases by about 30%. Cholestasis also increases the recovery of porphyrins in the urine, although light treatment of ligated rats further increases urinary porphyrin excretion. The spectroscopic changes induced by irradiation of sera of ligated rats are consistent with the formation of products that have the typical spectrum found in bronze baby syndrome patients, *i.e.* a reduced absorbance in the visible region and an increased absorption in near-UV and red spectral regions. The products responsible for the brown discoloration found in bronze baby syndrome seem to result from phototransformation of copper-porphyrins subsequent to an electron transfer between photoexcited bilirubin and the copper ion. (*Pediatr Res* 27: 22–25, 1990)

Abbreviation

BBS, bronze baby syndrome

The BBS was first described in 1972 by Kopelman *et al.* (1), who observed a newborn infant with a gray-brown discoloration after phototherapy for unconjugated hyperbilirubinemia. Subsequent reports (2–6) described the clinical aspects of this syndrome, whereas other studies attempted to clarify the biochemical mechanisms leading to BBS (7–9). In 1982, large amounts of porphyrins were demonstrated in sera of BBS patients (8). These were identified as Cu-uro-, Cu-copro-, and Cu-protoporphyrin (9). In addition, large amounts of porphyrins were detected in sera of adult and pediatric patients with cholestatic disorders (10). The proposed mechanism for the onset of BBS invokes, first, the presence of cholestasis, causing elevated copper and porphyrin concentrations in serum, and second, hyperbilirubinemia and phototherapy (9–11). Cu-porphyrins undergo photodestruction, sensitized by bilirubin, to products that have generalized absorption in the near UV and red spectral regions and therefore are responsible for the brown discoloration (9–11).

To shed further light on the mechanisms of development of this syndrome, we evaluated an animal model of BBS using Wistar rats subjected to ligation of the common bile duct. This strain of animals was chosen because in preliminary experiments we observed a consistent serum level of both conjugated and unconjugated bilirubin after common bile duct ligation. The results of these experiments were similar with those obtained by using heterozygous Gunn rats (12).

Received March 15, 1989; accepted August 3, 1989.
Correspondence and reprint requests Prof. F. F. Rubaltelli, Department of Pediatrics, University of Padova Via Giustiniani, 3 35128 Padova, Italy.

MATERIALS AND METHODS

Chemicals. Bilirubin IX was purchased from Serva (Heidelberg, FRG) and used without further purification. Its concentration was evaluated by absorbance measurements at 460 nm using a molar extinction coefficient 164 000 $M^{-1} cm^{-1}$ (ethanol solution) (13). All the free base porphyrins used were obtained from Porphyrin Products (Logan, UT) and converted into their Cu(II) derivatives by reaction with cupric acetate (14). SDS was the product of Merck (Darmstadt, FRG). All other chemicals were commercial products of at least analytical grade.

In vivo studies. In all our experiments, male Wistar rats (Ditta Morini, Reggio Emilia, Italy) between 3 and 9 mo old weighing between 250 and 350 g were used. Preliminary experiments showed that the body content of endogenous porphyrins is sex dependent and generally larger for older rats. The rats were housed in standard cages with free access to tap water and normal dietary food, except for a controlled low content of copper.

The rats were divided into four groups: *1*) Control rats were kept in metabolic cages, where urine and feces were collected at 24-h intervals. They were killed by exposure to ether, and blood and liver samples were quickly removed. The liver was stored at -17°C, whereas the blood was heparinized and the plasma was collected by 15-min centrifugation at 3000 rpm. *2*) Rats whose bile duct was ligated under pentothal anesthesia. No significant mortality resulted from this operation. After ligation, the rats were kept in metabolic cages for up to 96 h with urine and feces collection each 24 h. They were then killed and samples of blood and liver were removed and treated as described for the animals of group 1. *3*) Rats with ligated bile duct that were kept in metabolic cages for 48 h and then irradiated for 24 h as described below. At selected times after irradiation, the rats were killed and the blood and liver were treated as for group 1 animals. Urine was collected before and during irradiation. *4*) Rats that were kept in metabolic cages for 48 h and subsequently exposed to light for 24 h with collection of urine. After the phototherapy, the rats were killed and the blood and liver were treated as for group 1 animals.

Irradiation procedure. The rats in the metabolic cages were subjected to whole body irradiation by means of two superblue fluorescent lamps (Sylvania, Waltham, MA), which were symmetrically placed on opposite sides of the cage. The lamps had an emission maximum at 460 nm and a half-band width of 40 nm. The light fluence, at the level of the irradiated animals, was 1.2 W/m^2, as assessed with a radiometer. The fluence was periodically controlled during the phototreatment.

Analysis of porphyrins. Before analysis, the plasma and urine samples were diluted with known volumes of 2% aqueous SDS to obtain an absorbance less than 0.1 at both 400 and 620 nm. The solution was centrifuged for 10 min at 3000 rpm and the supernatant was collected and used for fluorescence analysis. Tissues (100–200 mg) were homogenized in a Potter vessel with

4 mL of 2% SDS. After 10 min centrifugation at 3000 rpm the supernatant was further diluted with 2% SDS to an absorbance less than 0.1 at 400 and 620 nm and then assayed spectrophotoflourimetrically.

The porphyrin content in biologic fluids and tissue homogenetes was quantified using an MPF 4 Perkin-Elmer (Pomona, CA) apparatus. All samples were excited at 400 nm and the fluorescence emitted at wavelengths at more than 600 nm was recorded. For fluorescence excitation measurements, the emission monochromator was set at 620 nm and the excitation spectrum in the 310- to 460-nm interval was recorded. The fluorescence intensity values were converted into porphyrin concentration by interpolation with a calibration plot built with known amounts of porphyrin. The values for porphyrin concentrations essentially refer to protoporphyrin IX. HPLC analysis of selected serum, urine, and tissue extracts showed that protoporphyrin accounted for at least 90% of total porphyrin recovery. Therefore, although there is some variability in the fluorescence yield among different porphyrins (15), the error introduced into our data should be minimal, because such differences tend to disappear when the individual porphyrins are in a monomeric state and in an identical microenvironment (16); in our samples, all porphyrins are embedded into the hydrophobic interior of SDS micelles. This assumption is supported by the observation that under our analytical conditions there is a linear relationship between porphyrin concentration and fluorescence intensity.

A major source of uncertainty in the estimation of porphyrins by fluorescence analysis of tissue homogenates is the presence of a wavelength-dependent scattering background (17). To evaluate this, we performed parallel analyses of our samples by fluorescence excitation at 395 nm and emission at 620 nm: a good agreement was found between the two sets of data. Under these conditions, a linear correlation is observed between fluorescence intensity and porphyrin concentration even in the presence of biologic materials (18).

In some experiments, the plasma content of Cu(II)-protoporphyrin IX was determined by HPLC according to Mascanzoni *et al.* (19). The HPLC apparatus was a Perkin-Elmer series 4 instrument, which was equipped with a Waters C-18 column (Waters Associates, Milford, MA). The serum (0.2 mL) was diluted 5-fold with methanol before loading on the column. The eluent system included two phases: initial elution was made with solvent A (acetonitrile/tetrabutylammonium phosphate/2.5 mM methanol, 3:2:1, vol/vol/vol) with a change to phase B (methanol/tetrabutylammonium phosphate 2.5 mM, 9:1, vol/vol) after 15 min. The eluate was continuously monitored for its absorbance at 400 nm and the area of the single peaks was estimated by a computer-controlled integrator.

Measurement of copper. The content of copper in the biologic fluids and the tissue homogenates (prepared by the procedures described previously) was measured by atomic absorption spectroscopy using a Perkin-Elmer 4000 AS spectrophotometer. The instrument had been previously calibrated with an aqueous solution of cupric acetate at a concentration of 0.1 ug/mL.

Measurement of bilirubin. The content of bilirubin in the biologic fluids and tissue homogenates in 2% aqueous SDS was determined by spectrophotofluorimetry. The samples containing the bilirubin as a monomer in the SDS micelles were excited at 460 nm and fluorescence emission was recorded between 480 and 600 nm. Bilirubin concentration was obtained by interpolation of the fluorescence intensity values with a suitable calibration plot built with known solutions of bilirubin in aqueous SDS (20).

Unconjugated bilirubin in the plasma was estimated by absorbance determinations at 546 nm after reaction with sulfanilic acid at 20–25 (21) Control studies showed that under our experimental conditions the absorbance is linearly related with the bilirubin concentration up to at least 25 mg/dL.

BBS case. A serum sample from a term infant who had developed BBS after phototherapy for hyperbilirubinemia was obtained on the 5th d of life. The serum was analyzed for bilirubin and porphyrin content by the above specified procedure before and after irradiation of serum with the superblue lamps.

Statistical analysis. Student's *t* test for grouped data was used to compare the values found in ligated and control rats.

RESULTS

In vivo studies. The concentrations of porphyrins, bilirubin, and Cu(II) ions from the plasma, urine, and liver of control Wistar rats (group 1) were in agreement with literature data (Table 1) (22). The corresponding values for irradiated rats (group 4) showed no significant differences. In Figures 1–3 we report the porphyrin concentrations in bile-duct ligated (group 2) and ligated plus irradiated (group 3) rats. It is evident that after irradiation, the plasma porphyrin concentration increases, whereas there is a decrease of the concentration in the liver. It is important to note that all the concentrations from urine have been estimated for individual animals during the 24 h subsequent to irradiation and the equivalent time period for bile duct-ligated unirradiated rats. The same groups of animals were kept in metabolic cages throughout the experiment. However, for plasma and liver, the individual animals were killed at predetermined times before analysis.

HPLC analyses of plasma taken from rats at 48 h after ligation of the bile duct showed that about 20% of protoporphyrin IX

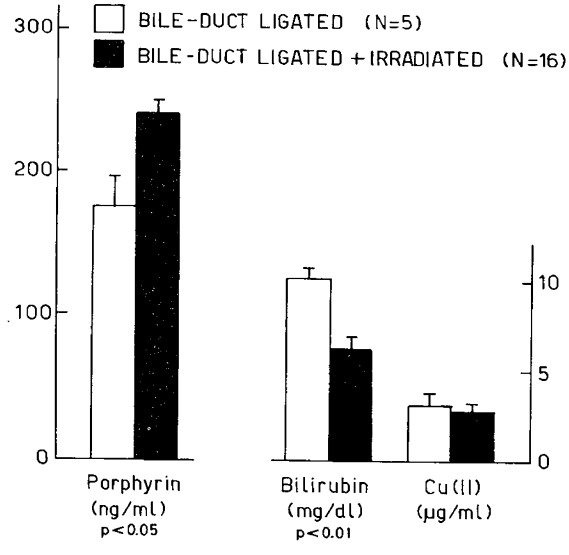

Fig. 1. Concentrations of porphyrin, bilirubin, and Cu(II) ions in plasma samples of rats 72 h after bile duct ligation (group 2) and in similar rats irradiated with superblue lamps for the last 24 h (group 3).

Table 1. *Concentration of porphyrin, bilirubin, and Cu(II) ions in plasma, urine, and liver samples of control rats**

Rats (*n*)	Porphyrin	Bilirubin	Cu(II)
Plasma (6)	24.0 ± 9.4 (ng/mL)	0.2 ± 0.06 (mg/dL)	1.2 ± 0.2 (μg/mL)
Urine (8)	0.10 ± 0.02 (mg/24 h)	5.7 ± 0.94 (mg/24 h)	0.41 ± 0.06 (μg/24 h)
Liver (3)	39.5 ± 0.5 (ng/g)	2.7 ± 0.4 (mg/g)	2.9 ± 0.6 (μg/g)

* All values represent mean SD.

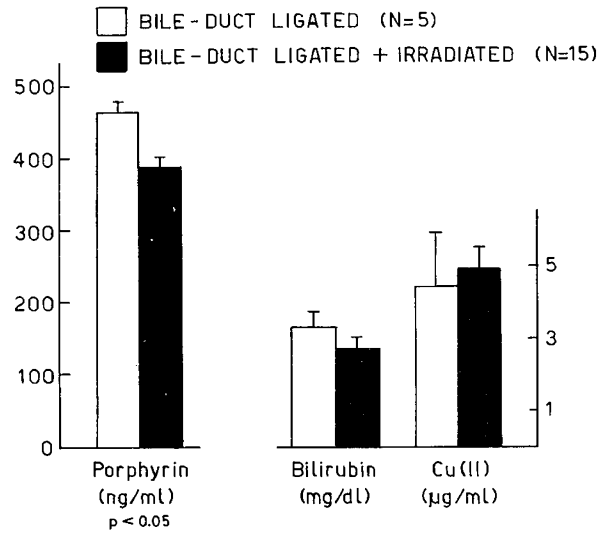

Fig. 2. Concentrations of porphyrin, bilirubin, and Cu(II) ions in livers of rats 72 h after bile duct ligation (group 2) and in similar rats irradiated with superblue lamps for the last 24 h (group 3) rats.

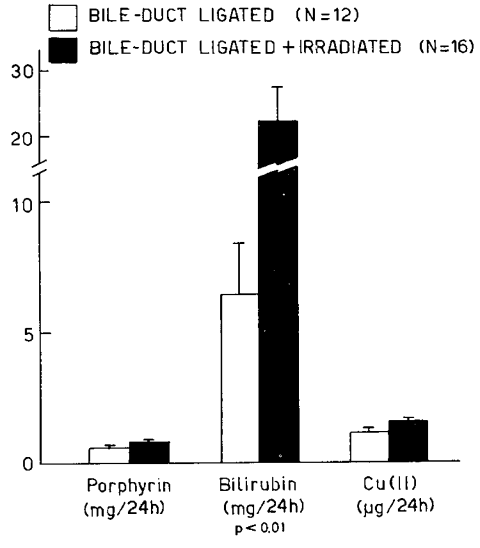

Fig. 3. Concentrations of porphyrin, bilirubin, and Cu(II) ions in urine of rats 72 h after bile duct ligation (group 2) and in similar rats irradiated with superblue lamps for the last 24 h (group 3) rats.

had been converted into the corresponding Cu(II)-protoporphyrin, whereas only traces of Cu(II)-protoporphyrin were found in plasma of control rats. After 24 h irradiation of the ligated rats, the plasma content of Cu(II)-protoporphyrin increased by about 30%. Again, no formation of Cu(II)-protoporphyrin was detected upon blue light irradiation of rats whose bile duct had not been ligated.

In vitro studies. The plasma obtained from four rats whose common bile duct had been ligated for 48 h were pooled and irradiated by the superblue lamps. The absorption spectra recorded at 3, 9, and 15 h after the beginning of the irradiation are shown in Figure 4. The content of bilirubin decreased from 9.32 to 5.73 mg/dL, whereas the content of protoporphyrin (0.29 ng/mL) remained essentially unchanged.

Exposure of the plasma to light brings about a substantial decrease of the bilirubin concentration, which is in agreement with the known photolability of this compound (13). This fact explains the photo-induced decrease of absorbance at wavelengths less than 550 nm (Fig. 4). At the same time, one can observe a gradual enhancement of the absorbance in the red spectral regions (at >580 nm). Essentially identical spectral modifications have been previously obtained upon irradiation of the bilirubin/Cu(II)-protoporphyrin system (11) and were ascribed to a photosensitized degradation of Cu(II)-protoporphyrin. Actually, the content of the latter compound was decreased in irradiated plasma from 0.08 to 0.03 ng/mL.

Analogously, with *in vitro* 24-h irradiation of the serum of a neonate who had developed BBS, we observed the appearance of a dark brown color with absorption spectral changes overlapping those reported in Figure 4. These modifications were accompanied by the decrease of both bilirubin (15.44 to 9.93 mg/dL) and Cu(II)-protoporphyrin (0.17 to 0.09 ng/mL). The photoprocesses were accelerated by the addition of fresh bilirubin to the irradiated serum, whereas the addition of either protoporphyrin or its cupric derivative had no significant effect.

DISCUSSION

The exact nature of the pigments responsible for the bronze color of the skin in neonates developing BBS is still debatable. The involvement of photoisomerization and photooxidation products of bilirubin (7, 23) and biliverdin-related compounds (24) has been proposed. However, previous studies from our laboratory (8, 9) pointed out the presence of abnormally high serum concentrations of copper-porphyrins in those hyperbilirubinemic neonates developing BBS upon phototherapy; blue-light irradiation of these porphyrins in the presence of bilirubin yields brown photoproducts whose spectral features closely resemble those typical of sera isolated from BBS patients (10, 24).

Copper-porphyrins can be spontaneously formed in the presence of large amounts of Cu(II) ions and free base porphyrins due to the large affinity of the tetrapyrrolic macrocycle for a variety of metal ions (14). The excess Cu(II) ions could overcome the binding capacity of endogenous serum and liver proteins. Such a mechanism for the onset of BBS is in agreement with the known deranged liver function and cholestasis in BBS patients (6, 23), whereas the need for the simultaneous presence of cholestasis, hyperbilirubinemia, and phototherapy would explain the rare occurrence of this syndrome.

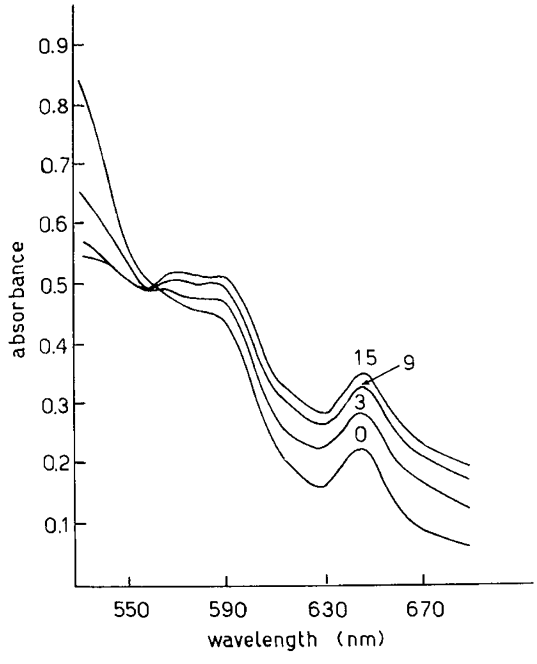

Fig. 4. Visible absorption spectrum of pooled plasma samples obtained from four rats 48 h after ligation of common bile duct. The plasma samples were irradiated for 0, 3, 9, or 15 min.

To check the validity of our hypothesis, we developed an animal model where rats were artificially made cholestatic and hyperbilirubinemic. Under these conditions, the accumulation of both copper and porphyrins (mainly protoporphyrin) in the serum and liver is observed. HPLC analysis of the porphyrins extracted from the sera indicates that about 20–22% of the accumulated protoporphyrin is transformed into the Cu derivative. Similarly, significant amounts of Cu(II)-protoporphyrin and smaller amounts of Cu(II)-uroporphyrin are present in the serum of a BBS patient. No detectable amounts of copper-porphyrins are found in the sera of either normal or hyperbilirubinemic but not cholestatic neonates (10). The data obtained in the present investigation clearly show that the ligation of the common bile duct is necessary for the formation of Cu(II)-porphyrins. Only small amounts of the porphyrins are eliminated in the urine (Fig. 3), which is in agreement with the predominant formation of hydrophobic derivatives, such as protoporphyrin. Actually, poorly water-soluble porphyrins are largely eliminated via the bile-gut pathway, whereas only very polar porphyrins (such as uroporphyrin) are cleared from the organism in the urine (25).

The spectroscopic changes induced by irradiation of sera obtained from both ligated rats and a BBS patient are consistent with a decrease in the concentration of bilirubin and the formation of products with an increased absorption in the near-UV and red spectral region. Similar observations were made by other authors (24). The photodegradation of bilirubin is a well-described process (26) leading to photooxidized pyrroles and bilirubin isomers, all of which have a reduced absorbance in the visible region and lack any absorbance at wavelengths of more than 600 nm. However, porphyrin photoproducts with new absorption bands in the red region have been reported (27); in particular, oxidized polymeric derivatives of porphyrins display a brown color. Our previous *in vitro* studies (11) suggest that the phototransformation of copper-porphyrins is sensitized by the initially photoexcited bilirubin.

Thus, the HPLC analysis of irradiated plasma demonstrates that porphyrin itself is rather photostable, whereas Cu(II)-protoporphyrin undergoes a significant drop in concentration. It is likely that the observed formation of brown products reflects a photodegradation of Cu(II)-protoporphyrin subsequent to an electron transfer between photoexcited bilirubin and the copper ion (11).

In conclusion, the studies with our animal model indicate that the induction of cholestasis by bile duct ligation causes a large increase in plasma and liver content of porphyrins, including the formation of Cu(II)-protoporphyrin. Subsequent irradiation increases the plasma concentration of Cu(II)-protoporphyrin with the simultaneous appearance of spectroscopic changes reproducing those observed upon irradiation of the sera obtained from BBS neonates.

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