Severe Neonatal Hyperbilirubinemia in Crigler-Najjar Syndrome Model Mice Can Be Reversed With Zinc Protoporphyrin

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Neurotoxic bilirubin is solely conjugated by UDP-glucuronosyltransferase (UGT) 1A1. Due to an inadequate function of UGT1A1, human neonates develop mild to severe physiological hyperbilirubinemia. Accumulation of bilirubin in the brain leads to the onset of irreversible brain damage called kernicterus. Breastfeeding is one of the most significant factors that increase the risk of developing kernicterus in infants. Why does the most natural way of feeding increase the risk of brain damage or even death? This question leads to the hypothesis that breast milk-induced neonatal hyperbilirubinemia might bring certain benefits to the body. One of the barriers to answering the above question is the lack of animal models that display mild to severe neonatal hyperbilirubinemia. A mouse model that develops neonatal hyperbilirubinemia was previously developed by a knockout of the Ugt1 locus. Deletion of Ugt1a1 results in neonatal lethality from bilirubin neurotoxicity. Bilirubin is the end product of heme catabolism in which heme oxygenase-I is largely involved. When zinc protoporphyrin, an inhibitor of heme oxygenase I, was administered to newborn Ugt1−/− mice, serum bilirubin levels dropped dramatically, rescuing the mice from bilirubin-induced neonatal lethality. Zinc protoporphyrin-treated Ugt1−/− mice developed normally as adults capable of reproducing, but their newborns showed even more severe hyperbilirubinemia. Microarray analysis of the hyperbilirubinemic livers indicated that a number of genes associated with nucleotide, transport, and immune response were significantly down-regulated in a serum bilirubin level-dependent manner. Conclusion: Our study provides an opportunity to advance the development of effective therapeutics to effectively and rapidly prevent bilirubin-induced toxicity. Neonatal hyperbilirubinemia has various impacts on the body that could be driven by the antioxidant property of bilirubin. (Hepatology Communications 2017;1:792–802)

Introduction

Newborn children’s breathing and excessive oxygen intake stimulates red blood cell hemolysis. Heme is a porphyrin that is coordinated with Fe(II) and is the oxygen-carrying portion of hemoglobin in red blood cells. Hemolysis results in the release of hemoglobin and heme from red blood cells. The reticuloendothelial system participates in oxidative degradation of heme by heme oxygenase-I (HO-I) to form biliverdin,(1) which is immediately reduced to bilirubin by bilirubin reductase (Fig. 1). (2)
Whereas biliverdin is hydrophilic and nontoxic, bilirubin is highly hydrophobic and neurotoxic. In mammals, bilirubin is mainly detoxified by UDP-glucuronosyltransferase (UGT) 1A1, which mediates the glucuronidation of bilirubin to form hydrophilic and nontoxic mono- and di-glucuronides. In addition, bilirubin can be subject to partial metabolism by cytochrome P450s (CYPs), which form oxidized metabolites.

Newborn children develop mild hyperbilirubinemia, called jaundice, due to inadequate expression of UGT1A1 in the liver and small intestine. Because total serum bilirubin (TSB) levels often fall without additional treatment within several weeks after birth, such conditions are physiological. In contrast, genetic deficiencies that leave the UGT1A1 gene defective cause severe neonatal hyperbilirubinemia (SNH). Crigler-Najjar syndrome type I (CN-I) and type II (CN-II) are inherited diseases in which UGT1A1 is completely or severely deficient, respectively. Whereas CN-II can be treated by chemical induction of the UGT1A1 gene by agents such as phenobarbital, the treatment for CN-I is more complex and often requires transplantation of the liver or hepatocytes. The extremely high TSB levels in CN-I lead to filtration of bilirubin into brain tissue. Due to the neurotoxic property of bilirubin, its accumulation in brain tissue can lead to acute bilirubin encephalopathy, followed by the more chronic form called kernicterus. Although there are examples of patients with CN-I who have reached adulthood without requiring a liver transplant, the syndrome is often lethal if immediate and drastic clinical intervention is not available. The current therapies to interrupt the steady increase in SNH, such as extensive phototherapy, exchange transfusion, or liver transplant, are designed to eliminate bilirubin after its accumulation. An alternative approach is to prevent the accumulation of bilirubin. To examine this possibility, animal models with deficiencies in UGT1A1 expression can be used to develop new tools aimed at preventing bilirubin accumulation.

When the mouse Ugt1a1 gene and the Ugt1 locus are rendered inactive in Ugt1<sup>−/−</sup> mice, newborn Ugt1<sup>−/−</sup> mice develop SNH composed of unconjugated bilirubin, resulting in accumulation of bilirubin.
in the brain and lethality within 1-2 weeks after birth.\(^{(9)}\) Disruption of the \(Ugt1\) locus generated a phenotype that resembles CN-I disease. It was subsequently shown that the lethality associated with neonatal unconjugated bilirubin accumulation in \(Ugt1^{-/-}\) mice could be prevented following the introduction of the human UGT1 locus and the \(UGT1A1\) gene.\(^{(10)}\) More recently, Muro’s group generated another CN-I mouse model by inserting a premature stop codon in the \(Ugt1a1\) gene\(^{(11)}\) and could thus prevent bilirubin-induced lethality with gene therapy. When they injected an adenovirus expressing human \(UGT1A1\) complementary DNA (cDNA), the neonatal mice reached adulthood with serum bilirubin levels similar to those in wild-type mice.\(^{(11)}\) Although encouraging, gene therapy in humans is still in the experimental phases.

In the absence of functional UGT1A1, blockage of bilirubin production or accelerating alternative routes of bilirubin metabolism are the available choices to prevent the toxicity associated with SNH. To examine these choices, we assessed the impact of oxidative metabolism on bilirubin clearance and the potential of blocking bilirubin accumulation. In this study, we examined the potential of \(\beta\)-naphthoflavone and protoporphyrins (PPs) to block bilirubin accumulation. Due to the structural similarity between PPs and heme (Fig. 1), PPs have been exploited as HO-I inhibitors.\(^{(12)}\) Following chemical treatment, bilirubin accumulation was assessed along with alterations in lethality.

Materials and Methods

ANIMALS AND CHEMICAL TREATMENTS

All mice used in the present study were housed under a 12-hour light/12-hour dark cycle at 21°C-23°C and received food and water \(ad\) \(libitum.\) Animal experiments were approved by the Animal Experimentation Committee of Kitasato University. The \(Ugt1\) knockout (KO) CN-I model mice as well as the \(Tg\) (\(UGT1A1^{+}\)) \(Ugt1^{-/-}\) (humanized \(UGT1\) [h\(UGT1\)]) mice were developed previously in a C57BL/6 background.\(^{(9,10,13)}\) Newborns were treated with zinc protoporphyrin (ZnPP, \(1-100\) \(\mu\)mol/kg/day, subcutaneously) from the second day after birth for 12 days. Blood was obtained from the submandibular vein and was then centrifuged at 3,000 \(g\) for 5 minutes to obtain serum. Total serum bilirubin levels were quantified using a Bilirubinometer (B-105N; Erma, Tokyo, Japan). Mice were anesthetized by diethyl ether inhalation, and the livers were isolated and rinsed in cold 1.15% KCl and stored at \(-80^\circ C.\)

MICROARRAY AND GENE ONTOLOGY ANALYSIS

Livers from 2-day-old mice were used for microarray analysis. Heterozygous \(Ugt1^{+/+}\) mice were bred to generate \(Ugt1^{+/+}\), \(Ugt1^{+/-}\), and \(Ugt1^{-/-}\) (KO) mice. KO mice treated with ZnPP that matured to adults were further bred to produce \(Ugt1^{-/-}\) (KOKO) mice. Three livers from each group (\(Ugt1^{+/+}\), \(Ugt1^{+/-}\), \(KO\), and \(KOKO\)) were pooled. Total RNA was purified using a NucleoSpin RNA cleanup kit. RNA integrity was evaluated with a Nanodrop LITE Spectrophotometer. cDNA was synthesized from 500 ng of total RNA, amplified, fragmented, and labeled using a GeneChip WT PLUS Reagent kit according to the manufacturer’s instructions. The biotinylated targets were hybridized to Mouse Gene 2.0 ST Array (Affymetrix, Santa Clara, CA) containing 35,240 transcripts. After hybridization, the arrays were washed and stained using a GeneChip Hybridization, Wash and Stain Kit according to the manufacturer’s instructions. Afterward, the arrays were scanned with a GeneChip scanner 3000 7G (Affymetrix). GeneChip data quality control was performed using Expression Console Software, ver. 1.4, and raw data were preprocessed based on the Robust Multi-array Average algorithm for normalization and summarization.

Gene ontology (GO) analysis was conducted with a Cytoscape plugin BiNGO program.\(^{(14)}\) GO analysis was also conducted using DAVID bioinformatics resources.\(^{(15)}\)

QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA was extracted from tissues with Trizol reagent (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from total RNA using ReverTra Ace (TOYOCO, Osaka, Japan) according to the manufacturer’s protocol. Primers for mouse cyclophilin and human glyceraldehyde 3-phosphate dehydrogenase had been developed.\(^{(16,17)}\) Sense and antisense oligonucleotide primers for mouse actin alpha-1 (5’-TTC CGT CTG ACC GCA TGC A-3’ and 5’-GGG CGA TGA TCT TGA TCA-3’) and human
ACTIN alpha-1 (5'-CCC GCC CAG AAA CTA GAC AC-3' and 5'-GAC CCA TAC CGA CCA TGA CG-3') were established with the Primer Blast program (National Institutes of Health). Polymerase chain reaction conditions have been described. (18) Expression of cyclophilin and glyceraldehyde 3-phosphate dehydrogenase messenger RNA was used as an internal control for cDNA quantity and quality.

CELL CULTURE AND TREATMENTS

Human hepatoma HepG2 cells were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). HepG2 cells were cultured as reported. (19) HepG2 cells were seeded at a density of 1 x 10^5/well in 12-well plates with the culture medium containing the indicated concentration (µM) of bilirubin and vitamin E (α-tocopherol). Twenty-four hours after incubation of the cells at 37°C, total RNA was isolated from HepG2 cells using TRIzol reagent according to the manufacturer's instructions.

STATISTICS

Data were presented as means ± SD. Statistical analyses were conducted using the unpaired t test or Dunnett's test. A value of P < 0.05 was considered statistically significant.

Results

CHEMICAL TREATMENT OF CN-I MODEL MICE

Because there is no functional UGT1A1 in Ugt1^-/- mice, these mice develop SNH with accumulation of bilirubin in brain tissue, which was identified as kernicterus. Due to the onset of kernicterus, SNH results in 100% fatality of newborn mice. (9) Although bilirubin is metabolized mainly through glucuronidation, it has been shown that oxidative metabolism by CYPs can also facilitate bilirubin metabolism. In our preliminary study, treatment of Ugt1^-/- newborns with β-naphthoflavone, an inducer of CYPs, slightly extended their survival (Supporting Fig. S1). Because HO-I is involved in the catabolism of heme, it was hypothesized that HO-I inhibitors could suppress the formation of bilirubin during the neonatal period and prevent the infiltration of bilirubin into the brain. (20)

When Ugt1^-/- mice were treated with low-dose ZnPP (1-10 µmol/kg/day) from 2 to 14 days after birth, none of the treated mice survived for more than 11 days. In contrast, Ugt1^-/- mice survived when they were treated with ZnPP at a higher dosage (100 µmol/kg/day) (red line in Fig. 2). Serum bilirubin levels in the ZnPP-treated mice were less than 10.0 mg/dL at day 12, which was lower than the TSB levels in hUGT1 mice at that same age (Fig. 3). Even though the chemical treatment was discontinued at 14 days after birth, the Ugt1^-/- mice survived for more than...
monitored, we found that the levels reached 13.4 Ugt1−/− mice that were born from Ugt1+/− mice, and Ugt1−/− mice that were born from Ugt1−/− mice are shown. (B) Blood was isolated from the Ugt1−/− mice that were born from Ugt1+/− mice at 29, 36, 43, 50, and 57 days after birth, and bilirubin levels were determined. *, $P < 0.05$ and **, $P < 0.01$ in analysis of variance with post-hoc Tukey HSD test. n = 33 (control), n = 3 (KO mice), and n = 4 (KOKO mice) in panel A; n = 6 in panel B. Data are mean ± SD. KO and KOKO indicate Ugt1−/− mice that were born from Ugt1+/− female mice and Ugt1−/− mice that were born from Ugt1−/− female mice, respectively. Abbreviation: HSD, honest significant difference.

21 days. When dosing entered the second week of treatment, ZnPP treatment (100 μmol/kg) could be administered every other day instead of every day (blue line in Fig. 2). At 22 days after birth, the mean TSB levels were 8.0 ± 3.0 mg/dL (Fig. 3).

ADULT CN-I MODEL MICE AND NEWBORNS FROM CN-1 MICE

Lethality associated with Ugt1−/− mice has been reported.9,11 However, little is known about the phenotype of adult Ugt1−/− mice because the genotype is lethal. In our study, the administration of ZnPP to Ugt1−/− mice prevented neonatal lethality in a total of 20 Ugt1−/− mice (12 male, 8 female). Body weight of 22-day-old Ugt1−/− mice that were treated with ZnPP was 7.6 ± 1.1 g, which is lower than determined in same-age Ugt1+/− littermates (Fig. 4A). Body weight of Ugt1−/− mice gradually increased, and the weights of 2-month-old male and female Ugt1−/− mice were 22.3 ± 1.5 g and 20.5 ± 0.6 g, respectively, which is still lower than wild-type mice. When serum bilirubin levels were monitored, we found that the levels reached 13.4 ± 1.6 and 15.3 ± 2.3 mg/dL in 2-month-old male and female Ugt1−/− mice, respectively (Fig. 4B). Except for one

FIG. 4. Body weight and serum total bilirubin levels in Ugt1−/− mice. Neonatal Ugt1−/− mice were treated with ZnPP (Fig. 2). (A) Body weights of 22-day-old control mice, Ugt1−/− mice that were born from Ugt1+/− mice, and Ugt1−/− mice that were born from Ugt1−/− mice are shown. (B) Blood was isolated from the Ugt1−/− mice that were born from Ugt1+/− mice at 29, 36, 43, 50, and 57 days after birth, and bilirubin levels were determined. *, $P < 0.05$ and **, $P < 0.01$ in analysis of variance with post-hoc Tukey HSD test. n = 33 (control), n = 3 (KO mice), and n = 4 (KOKO mice) in panel A; n = 6 in panel B. Data are mean ± SD. KO and KOKO indicate Ugt1−/− mice that were born from Ugt1+/− female mice and Ugt1−/− mice that were born from Ugt1−/− female mice, respectively. Abbreviation: HSD, honest significant difference.

FIG. 5. Treatment of Ugt1−/− mice born from Ugt1−/− female mice with ZnPP. The survival curve of Ugt1−/− mice born from Ugt1−/− female mice (KOKO) is shown (red line) and is compared to that of Ugt1−/− mice born from Ugt1+/− female mice (KO) (black line). The Ugt1−/− mice born from Ugt1−/− female mice (KOKO) were treated with ZnPP (100 μmol/kg/day, subcutaneously) at days 2-14 after birth (blue line); n = 20 (black line), n = 10 (red line), and n = 5 (blue line).

male mouse, all the ZnPP-treated Ugt1−/− mice reached 2 months of age.

The successful treatment of Ugt1−/− mice with ZnPP to avoid SNH-induced lethality resulted in adult mice that could reproduce. Each delivery resulted in three to nine newborns per litter, and all were CN-I

FIG. 6. Quantitative-PCR analysis of actin alpha-1 expression in the livers. Livers were isolated from 2-day-old WT mice, Ugt1+/− mice, Ugt1−/− mice that were born from Ugt1+/− female mice, and Ugt1−/− mice that were born from Ugt1−/− female mice (n = 3 each). Livers in each group were pooled, cDNA was synthesized, and expression levels of actin alpha-1 were determined by real-time PCR. Relative expression levels are shown in the figure. Data (mean ± SD) were pooled from five experiments. KO and KOKO indicate Ugt1−/− mice that were born from Ugt1+/− female mice and Ugt1−/− mice that were born from Ugt1−/− female mice, respectively. Abbreviations: mRNA, messenger RNA; PCR, polymerase chain reaction; WT, wild-type.
as highlighted previously with the Ugt1<sup>+/−</sup> background. When we compared the survival curves of neonatal Ugt1<sup>+/−</sup> that were generated from crosses between Ugt1<sup>1+/−</sup> male and female mice and crosses from ZnPP-treated male and female adult Ugt1<sup>+/−</sup> mice, the survival curve of offspring from ZnPP-treated mice was much shorter than from Ugt1<sup>1+/−</sup> mice (Fig. 5, red line). Two days after birth, the TSB levels from neonatal Ugt1<sup>+/−</sup> mice generated from adult ZnPP-treated mice (14-15 mg/dL) were considerably higher than the newborns generated from Ugt1<sup>1+/−</sup> breeders (2-10 mg/dL). We anticipate that the accelerated lethality was attributed to higher unconjugated bilirubin levels right after birth and was due to the incapability of bilirubin glucuronidation during pregnancy in the Ugt1<sup>+/−</sup> female mice.

**TABLE 1. TOP 15 TRANSCRIPTS THAT WERE REDUCED IN THE LIVER OF UGT1<sup>−/−</sup> MICE (KOKO) IN MICROARRAY ANALYSIS**

| Cluster ID | Gene Symbol | Description | Ugt1<sup>+/−</sup> | Ugt1<sup>−/−</sup> (KO) | Ugt1<sup>−/−</sup> (KOKO) |
|------------|-------------|-------------|-------------------|------------------------|------------------------|
| 17513995   | Acta1       | actin, alpha 1, skeletal muscle | −3.79             | −29.93                 | −36.18                 |
| 17312186   | Psco        | prostate stem cell antigen       | −41.83            | −31.92                 | −28.79                 |
| 17251133   | Myh8        | myosin, heavy polypeptide 8      | −6.07             | −26.96                 | −23.11                 |
| 17318045   | Cyp11b1     | cytochrome P450, family 11, subfamily b1 | −15.20          | −18.95                 | −17.71                 |
| 17517831   | Cyp11a1     | cytochrome P450, family 11, subfamily a1 | −14.58          | −15.91                 | −15.15                 |
| 17322075   | Krt4        | keratin 4                          | −13.38            | −12.95                 | −14.42                 |
| 17343962   | Cyp21a1     | cytochrome P450, family 21, subfamily a1 | −12.01          | −11.27                 | −12.67                 |
| 17517380   | Sln         | sarcolinip                          | −5.71             | −15.29                 | −12.52                 |
| 17223916   | Myl1        | myosin, light polypeptide 1        | −3.30             | −12.30                 | −11.95                 |
| 17496211   | Atp2a1      | ATPase, Ca<sup>++</sup> transporting | −3.39            | −13.47                 | −11.07                 |
| 17290603   | Actn2       | actinin alpha 2                     | −3.41             | −12.84                 | −10.34                 |
| 17548055   | LOC630751   | interferon-inducible guanosine     | −3.66             | −1.44                  | −9.50                  |
| 17232453   | Trdn        | triadin                              | −3.67             | −8.56                  | −8.62                  |
| 17243910   | Mybpc1      | myosin binding protein C, slow-type | −3.32             | −16.82                 | −8.61                  |
| 17445565   | Gm15611     | predicted gene 15611                | −1.92             | −2.24                  | −8.41                  |
Regardless, the newborns from the *Ugt1<sup>+/−</sup>* adults responded well to ZnPP treatment (blue line in Fig. 5) and reached adulthood. The body weight of 22-day-old *Ugt1<sup>−/−</sup>* mice generated from adult ZnPP-treated mice averaged 6.5 ± 0.7 g, which is significantly lower than wild-type mice (Fig. 4A).

**MICROARRAY AND GO ANALYSIS OF NEWBORN Ugt1<sup>−/−</sup> MICE**

The accelerated lethality that was apparent in the newborns from adult *Ugt1<sup>−/−</sup>* mice indicates that the elevated unconjugated bilirubin levels induce physiological toxicity that may include additional systemic processes in addition to those that are linked to the onset of chronic encephalopathy. To examine this possibility, we examined differences in liver gene expression in 2-day-old mice. The *Ugt1<sup>−/−</sup>* neonates generated from *Ugt1<sup>+/+</sup>* adult mice are referred to as KO mice, whereas the *Ugt1<sup>−/−</sup>* neonatal mice generated from adult (ZnPP-treated) *Ugt1<sup>−/−</sup>* mice are referred to as KOKO mice. When expression levels were determined, 598 genes with over a 2-fold change were observed, with 203 profiling as up-regulated and 395 being down-regulated. The quality of our microarray analysis was confirmed by quantitative polymerase chain reaction analysis of the *actin alpha-1* gene (Fig. 6). Because the number of genes and the fold changes were significantly greater with negatively regulated genes, we examined these transcripts and linked them through GO analysis (Fig. 7). Impressively, gene expression in KO and KOKO mice showed attenuated homeostatic processes affecting immune response, muscle contractility, transport processes, and metabolic function. From the list of the most significantly repressed genes (Table 1), such as those tied to muscle contraction (i.e., *Acta1, Myb8, My11*) and Cd<sup>2+</sup> transport (i.e., *Sln, Atp2a1*), we can speculate that elevated TSB levels have a significant effect on cardiac function. Other key physiological processes as listed in the GO analysis (Table 2), such as key oxidative–reduction processes, support of the immune and inflammatory response, cholesterol metabolism, and downstream steroid production, were all compromised. Meanwhile, the number of genes and the fold changes were much less with positively regulated genes (Table 3). The GO mapping analysis further indicated that there was less impact of the positively regulated genes on biological processes (Fig. 8). Although the early actions of SNH in newborns have been shown to induce central nervous system toxicity, these studies indicate that
important systemic processes essential for neonatal development and overall health are influenced by early elevations in TSB immediately after birth.

REGULATION OF ACTIN-alpha 1 EXPRESSION IN HepG2 CELLS

Although bilirubin is neurotoxic, it is a potent antioxidant. To understand the involvement of antioxidant effects on the down-regulation of actin-alpha 1 expression in hepatic cells (Table 1; Fig. 6), human hepatoma HepG2 cells were treated with bilirubin and another antioxidant, vitamin E (α-tocopherol). In HepG2 cells, bilirubin concentration dependently reduced the expression of ACTIN α1 (Fig. 9A). A similar reduction in the expression of ACTIN-α1 was also observed in the α-tocopherol-treated HepG2 cells (Fig. 9B). These data strongly indicate that the expression of

| Cluster ID | Gene Symbol | Description | Ugt1+/− | Ugt1+/− (KO) | Ugt1+/− (KOKO) |
|------------|-------------|-------------|----------|-------------|---------------|
| 17307305   | Phf11c      | PHD finger protein 11C | 3.03     | 1.01        | 10.38         |
| 17307318   | Setdb2      | SET domain, bifurcated 2 | 3.68     | 1.25        | 7.57          |
| 17441660   | Sds         | serine dehydratase | 2.08     | 1.17        | 5.01          |
| 17280062   | Lpin1       | lipin 1 | 2.98     | 1.04        | 4.58          |
| 17368521   |             |            | 1.65     | −1.13       | 3.94          |
| 17396238   |             |            | 1.14     | 1.62        | 3.91          |
| 17526707   | Zbtb16      | zinc finger and BTB domain containing 16 | 2.07     | 1.41        | 3.91          |
| 17370954   | Upp2        | uridine phosphorylase 2 | 1.38     | −1.08       | 3.62          |
| 17236100   |             |            | 1.03     | 1.01        | 3.45          |
| 17312066   |             |            | 1.36     | −1.13       | 3.45          |
| 17549958   |             |            | 1.03     | 1.01        | 3.45          |
| 17406186   | Tdo2        | Tryptophan 2,3-dioxygenase | 1.40     | 1.26        | 3.36          |
| 17364932   | Got1        | glutamate oxaloacetate transaminase 1 | 1.37     | −1.01       | 3.26          |
| 17330168   | BC100530    | cDNA sequence BC100530 | 3.06     | 2.51        | 3.21          |
| 17370339   | Olfr345     | olfactory receptor 345 | 1.64     | 3.21        | 3.20          |

FIG. 8. GO analysis of up-regulated genes in hyperbilirubinemic mice. GO analysis of genes that were up-regulated in hyperbilirubinemia mice was conducted using a BiNGO program. KO and KOKO indicate Ugt1−/− mice that were born from Ugt1+/− female mice and Ugt1+/− mice that were born from Ugt1+/− female mice, respectively. Underlined groups, such as Transport and Metabolic, are categories that were specifically affected in Ugt1−/− mice.
hepatic actin-alpha 1 is regulated by the antioxidative property of bilirubin.

**Discussion**

Severe neonatal hyperbilirubinemia develops in newborns due to a dramatic imbalance in the production and UGT1A1-mediated metabolism of bilirubin. If untreated, escalating levels of TSB can lead to acute and chronic forms of encephalopathy, culminating in severe brain damage, which is classified as kernicterus. Chronic forms of bilirubin-induced encephalopathy can leave children with lifelong physical disabilities, including mental retardation.\(^{21}\) Currently, there are no effective drug therapies to accelerate bilirubin metabolism or reduce bilirubin accumulation in cases of SNH. This is not a trivial clinical problem as this debilitating syndrome has been estimated to impact over 1 million children every year.\(^{22}\) Thus, the implementation of effective animal models to study the impact of SNH on molecular and cellular processes targeted by elevated TSB levels can be effectively leveraged for the development of new therapeutics designed to prevent bilirubin toxicity.

An alternative approach to prevent the buildup of unconjugated bilirubin in CN1 is to block its initial accumulation by inhibiting HO-1, the initial rate-limiting step in bilirubin production. Metalloporphyrins, derivatives of heme, are effective inhibitors of HO-1 and have been tested in mice to examine their potential to decrease bilirubin production. ZnPP is a naturally occurring metalloporphyrin, a potent inhibitor of HO-1, and has been shown in mice preloaded with heme to inhibit liver HO-1 activity following intragastric injections.\(^{23}\) Targeted deletion of the Ugt1 locus in Ugt1\(^{-/-}\) mice leads to newborn mice with nonhemolytic hyperbilirubinemia,\(^{9}\) a condition that closely resembles CN-1 because there is no functional UGT1A1. Although the phenotype associated with the Ugt1\(^{-/-}\) mice leads to rapid development of SNH, which is lethal within 1 week after birth,\(^{9}\) we demonstrated that subcutaneous injections of ZnPP were effective in reducing TSB levels and preventing lethality (Fig. 2). In hUGT1 mice that also develop SNH resulting from a developmental delay in UGT1A1 expression, induction of hemolysis by phenylhydrazine treatment to different-aged neonatal mice led to greater accumulation of TSB levels.\(^{10}\) For the youngest age group (7-8-day old), 100% of the mice developed seizures and died compared to only 10% in 13-day-old mice. These findings indicated that SNH is a prelude to central nervous system (CNS) damage within the first 2 weeks of life, demonstrating that brain tissue maturation can combat the impact of bilirubin in later stages of development. By delaying the rapid accumulation of TSB levels in ZnPP-treated Ugt1\(^{-/-}\) mice early in neonatal development, damaging levels of unconjugated bilirubin are not reached; maturation of brain tissue and the blood brain barrier then serve to block additional brain damage in the older mice. Thus, lowering TSB levels in this CN-1 model early in development protects brain tissue from damage, providing an opportunity to establish CNS defense against bilirubin toxicity.

Although the inability to metabolize bilirubin in newborns results in a clinical emergency, we have established the possibility that interrupting the production of bilirubin by blocking HO-1 activity may provide a unique mechanism for treating the most severe forms of hyperbilirubinemia. Once the appropriate dose of ZnPP was established for treating Ugt1\(^{-/-}\) mice, we achieved nearly a 100% survival rate, with all the neonatal mice reaching adulthood (Fig. 2). Although their TSB levels plateaued around 10 mg/dL as adults (Fig. 4B), the Ugt1\(^{-/-}\) mice showed no signs of bilirubin-induced toxicity and were capable of reproducing. However, compared to Ugt1\(^{+/+}\) mice, adult Ugt1\(^{-/-}\) mice weighed significantly less (Fig. 4A). This may indicate that bilirubin impacts insulin
sensitivity, which is directly correlated with obesity. For example, nonalcoholic fatty liver disease (NAFLD) is directly associated with obesity, insulin resistance, and metabolic syndrome.\(^{24,25}\) Weight gain and the progression to NAFLD and the more severe form of nonalcoholic steatohepatitis have been linked to a mismatch between oxidative stress and decreased antioxidant capacity. In children and adolescents, bilirubin has been shown to be inversely associated with insulin resistance, the metabolic syndrome, and NAFLD.\(^{26-28}\) These correlations between bilirubin accumulation against obesity and liver fibrosis may be due to the potent antioxidant properties associated with bilirubin.\(^{29}\) In addition, it is well known that coronary artery disease can result from changes in serum lipid oxidation, an event that can be protected in individuals with higher TSB levels, such as those observed in Gilbert’s syndrome. In the hyperbilirubinemic Gunn rat (j/j) model, adult mice at 24 weeks of age were 8% lighter than j/+ mice. When diabetes was induced by a single injection of streptozotocin, the j/j rats showed considerable resistance toward developing diabetes when compared to j/+ mice.\(^{30}\) More recently, hUGT1*28 mice, which express the human Gilbert’s allele and show elevated TSB levels, accumulated less fat as adults with lower serum glucose and insulin levels.\(^{31}\) These relationships indicate that elevated bilirubin levels in adult Ugt1\(^{-/-}\) mice may provide homeostatic protection from diet-induced oxidative stress, which leads to changes in insulin and glucose production that directly impacts fat production. Furthermore, breast feeding has been known to be one of the factors increasing TSB in infants.\(^{32-35}\) Breast milk-induced neonatal hyperbilirubinemia might be an essential condition for bringing beneficial advantages to the human body.

We have demonstrated that inhibition of HO-1 can be used to prevent lethality associated with SNH in newborn Ugt1\(^{-/-}\) mice. Although CNS toxicity in SNH is known as the primary damage in humans and replicable animal models, evidence is presented that systemic toxicities other than brain damage may be contributing to the irreversible damage that occurs in cases of extreme SNH shortly after birth. In line with epidemiologic studies in humans and corroborated in animal studies, hyperbilirubinemia in adults may be preventing diet-induced oxidative stress and its negative effect on weight gain, providing additional clues that hyperbilirubinemia can protect against the deleterious actions of insulin and glucose sensitivity. These studies may provide an opportunity to advance the development of effective therapeutics to rapidly and effectively prevent bilirubin-induced toxicities. In addition, an improved understanding of the underlying benefits of hyperbilirubinemia and the mechanisms leading to a reduction in diet-induced oxidative stress can be pursued.

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