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Metyrapone, an inhibitor of cytochrome oxidases, does not affect viability in a neuroblastoma cell model of bilirubin toxicity

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ABSTRACT

Background: Unconjugated hyperbilirubinemia may cause brain damage in infants, and globally remains a source of neonatal morbidity and mortality. A significant inter-individual variability in vulnerability to bilirubin toxicity remains largely unexplained. An enzyme located in mitochondria oxidizes bilirubin. We hypothesized that inhibiting bilirubin oxidation in human neuronal cell cultures exposed to bilirubin would increase cell death.
Methods: The ability of mitochondrial membranes from CHP-212 human neuroblastoma cells to oxidize bilirubin was verified by spectrophotometry. Intact cells in culture were exposed to bilirubin (75 μM) with or without metyrapone (250 μM) for 24 h, stained with Annexin-V and Propidium iodide and analyzed for apoptosis and necrosis by flow cytometry.
Results: Bilirubin caused a significant reduction of viability, from 84 ± 2.0% (mean ± SEM) vs 67 ± 2.7% ($p < 0.05$), but adding metyrapone to the bilirubin-exposed cells did not further impact cell viability. Metyrapone alone did not influence cell viability.
Conclusion: Herein we have shown that metyrapone does not increase cell death in neuroblastoma cells in culture exposed to bilirubin. Our results question the relationship between the oxidative mechanism evaluated by spectrophotometry and cell viability. Our findings add to the discussion on whether bilirubin oxidation represents a

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potentially important protective mechanism in neurons challenged by hyperbilirubinemia.

1. Introduction

Jaundice is the clinical condition most frequently requiring evaluation and treatment in the newborn, and the most common cause for hospital re-admission during the first week post partum [1]. Globally, neonatal jaundice is recognized as a source of significant neonatal morbidity, mortality, or life-long sequelae in some survivors [2]. Thus, studies from sub-Saharan Africa rank jaundice as the 2nd or 3rd cause of death in the newborn [2]. Kernicterus, the devastating sequelae of severe, unconjugated hyperbilirubinemia, manifests itself clinically as a tetrad of choreoathetoid cerebral palsy, high-frequency central neural hearing loss, palsy of vertical gaze, and dental enamel hypoplasia.

Although several important risk factors for Bilirubin Induced Neurological Dysfunction (BIND) [3] have been extensively studied, the determinants of vulnerability to and reversibility of this condition are still only partially understood [4]. In the clinical setting, we lack a specific test able to identify, prior to symptomatic neurotoxicity, a newborn likely to develop brain injury from high bilirubin levels. This is partly due to the variability in sensitivity between newborns to the neurotoxic effects of bilirubin. Hence, we need experimental models to aid us in clarifying how jaundice causes permanent brain damage in selected infants.

The neurotoxic properties of bilirubin cause cellular damage in all types of central nervous cells, but neurons appear to be most sensitive. It is possible that the cells possess various protective mechanisms, of which several candidates have been studied [5,6], and that cell survival depends on the orchestration of these mechanisms. Earlier reports have indicated that bilirubin is metabolized in the central nervous tissue by enzymatic oxidation in mitochondria, and have proposed that this may play a role in the brain's defense against bilirubin toxicity [7,8]. Studies have found such enzymatic activity in brain, as well as in other tissues [8,9]. Thus, the insoluble oxidase has been found i.a. on the inner mitochondrial membrane of brain cells in rats [8] and guinea pigs [9], confirming the oxidative metabolism of bilirubin in brain cells of those species. Previous studies have examined the regional distribution of this oxidative capacity, seeking to explain the patho-anatomical picture of kernicterus [8] The in vitro inhibition of this mitochondrial oxidase by ketoconazole [10] suggested a similarity to the cytochrome P450 oxidases. In earlier work from our group we have hypothesized a protective role of this oxidative action against bilirubin toxicity in the brain [10]. The purpose herein was to investigate whether inhibition of this apparent enzyme activity would affect cell survival in an in vitro model of bilirubin toxicity. We elected to use metyrapone, a drug with specific inhibitory effects on cytochrome P450 activity [11]. Cell death was quantitated by staining for apoptosis and necrosis followed by flow cytometric analysis. We postulated that if mitochondrial oxidation had a protective effect against bilirubin toxicity in this model, there would be an increase in cell death when cytochrome P450 was blocked by metyrapone.

2. Materials and methods

2.1. Bilirubin

Bilirubin (Sigma-Aldrich Corporation, St. Louis, MO, USA) was dissolved in 0.1 M NaOH and further diluted in de-ionized H_2O to a concentration of 5 mM. For the experiments with fixed b/a ratio, the bilirubin solution was mixed with 3.3 mM human serum albumin (HSA) (Sigma-Aldrich Corporation, St. Louis, MO, USA) to give a bilirubin/HSA stock solution of 2.5 mM, and a b/a ratio of 1.5. All handling of bilirubin was performed in dim light conditions to reduce photo-oxidation.

2.2. Cells

Neuroblastoma cells from the cell line CHP212 (ATCC-CRL2273) (American Type Culture Collection, Manassas, VA, USA) were used in the experiments. The CHP212 cells were cultured in 75 cm^2 flasks from Corning® (Sigma-Aldrich Corporation, St. Louis, MO, USA) in a growth medium of RPMI with L-glutamine (Roswell Park Memorial Institute medium, from BioWhittaker®, Lonza Group Ltd, Basel, Switzerland) and 10% Fetal Bovine Serum (BioWhittaker®, Cambrex Bio Science Verviers, Verviers, Belgium) and Penicillin/Streptomycin (BioWhittaker® Lonza Group Ltd, Basel, Switzerland) in a humidified, 5% CO_2, 37° C incubator. The medium was changed every second day and cells were sub-cultured to approximately 80% confluency every 4 to 5 days using 0.1% Trypsin (BioWhittaker®, Lonza Group Ltd, Basel, Switzerland). Cells were kept at 37° C, 5% CO_2 for all incubations, and in the bench at room temperature.

2.3. Cell staining

Annexin-V-Fluos, a marker of apoptosis, was purchased from Roche (Roche, Basel, Switzerland). Propidium iodide, which binds to intracellular DNA indicating cell membrane rupture, as seen in necrosis, was purchased from Sigma-Aldrich (Sigma-Aldrich Corporation, St. Louis, MO, USA).

2.4. Metyrapone

Metyrapone (Sigma-Aldrich Corporation, St. Louis, MO, USA) was dissolved in DMSO (Sigma-Aldrich Corporation, St. Louis, MO, USA) to a stock solution of 50 mM. A new stock solution was prepared for each experiment.

2.5. Assay of bilirubin oxidation by mitochondrial membranes

To verify that the neuroblastoma cells possessed the same bilirubin oxidizing ability as described in previous studies performed on rat and rabbit brains [8,10,12–17], cells were sub-fractionated to produce mitochondrial membrane fractions as described by Vakifahmetoglu [18].

Following the same procedure as Hansen and Allen [8], two hundred microliters of each mitochondrial membrane suspension was added to 2.5 ml of a 10 μM bilirubin solution and vigorously mixed. A 1 ml aliquot was centrifuged at approx. 20,000 *g* for 2 min to sediment the membranes. The optical density of the supernatant was recorded at 440 nm [19]. Metyrapone was added to a final concentration of 1 mM to half of the remaining samples of mitochondrial membranes in bilirubin solution, and all resulting suspensions were transferred to a water bath and incubated at 37.5 °C for 60 min. After 60 min a 1 ml aliquot was again removed from each reaction tube, immediately placed on ice, and centrifuged at approx. 20,000 *g* for 2 min. Optical density was again recorded at 440 nm, and the change in OD was calculated and corrected for background decay. The procedure was performed twice.

2.6. Flow cytometric cell death assay

Cells were seeded out in polystyrene plates (12 wells), $5 * 10^5$ cells/ml/well and incubated for 24 h at 37 °C in 5% CO_2. After 24 h, the supernatant was removed and 1 ml fresh RPMI without serum which had been preheated to 37 °C was added to each well. Cells were incubated for 30 min at 37 °C in 5% CO_2.

Cells were then incubated either with bilirubin 75 μM, metyrapone 250 μM, or both, and were kept for 24 h at 37 °C in 5% CO_2. The bilirubin concentration was adopted from our pilot studies with CHP2-12 cells to inflict a significant, but not lethal effect on cell viability. The chosen concentration of metyrapone was based on available literature on its uptake and enzyme inhibition assuming that it diffuses into human cells, reaching equilibrium with the metyrapone concentration in the medium [20]. On the following day, cells were trypsinated for 3–5 min at room temperature and then removed with cold RPMI without serum and centrifuged at 4 °C, washed again with Annexin binding buffer and re-suspended in phosphate buffered saline for flow cytometric analyses using a FACS Calibur Flow Cytometer, FC 500 (Beckman Coulter, Brea, CA, USA). The individual samples were stained with Annexin-V and Propidium iodide [21]

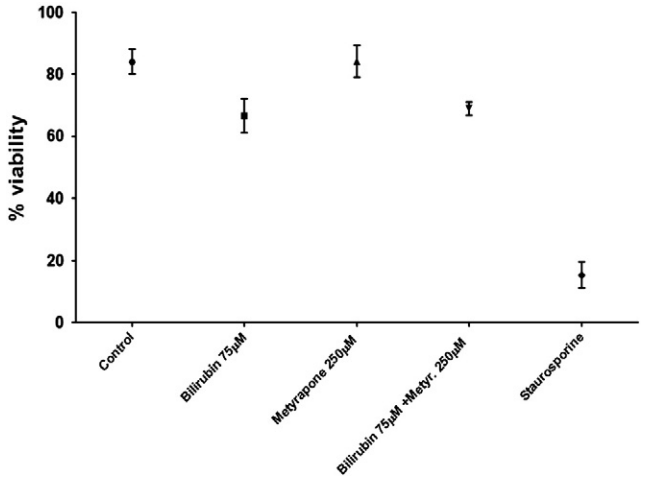

Fig. 1. Cells incubated with 75 μM bilirubin showed significant reduction in viability compared to controls ($p < 0.05$). However, adding 250 μM metyrapone, did not significantly change the viability in cells exposed to bilirubin ($p > 0.05$). Adding 250 μM metyrapone did not have any significant effect on the viability of control cells ($p > 0.05$). Staurosporine was used as positive control in the viability assay and caused massive cell death ($p < 0.05$).

and analyzed by flow cytometry for apoptosis and necrosis. Staurosporine (Sigma-Aldrich Corporation, St. Louis, MO, USA) was used for positive controls.

Early apoptosis was detected by Annexin staining of phosphatidylserine on the cell surface in the green spectrum (FL-1) at 485 nm bandwidth. Late apoptosis and necrosis were detected by Propidium iodide staining of DNA in the red spectrum (FL-2) at 405 nm bandwidth. 10^3 cells were counted from each sample and analyzed in CXP Analysis 2.0 (Beckman Coulter, Brea, CA, USA).

3. Results

3.1. Spectrophotometric assay

Adding metyrapone to the mitochondrial membrane suspension with bilirubin significantly inhibited the rate of oxidation as measured by decreased absorbance at 440 nm after 60 min. The membrane suspension without metyrapone had a bilirubin oxidation rate of 2.13 (SEM 0.02) nmol/min/mg protein; whereas the suspension with added metyrapone had a bilirubin oxidation rate of 1.01 (SEM 0.29) nmol/min/mg protein ($p < 0.05$), showing *i*) that mitochondria from these cells contain the bilirubin-oxidizing enzyme, and that *ii*) this enzyme is inhibited by metyrapone in vitro.

3.2. Flow cytometric assay

The statistical analysis was performed using independent sample T-tests between groups using PASW 18 (IBM Corporation, Armonk, NY, USA). The mean viability of the CHP2-12 cells in the metyrapone group remained unchanged compared to the control group, thus metyrapone alone had no effect on cell viability. Bilirubin 75 μM caused a significant ($p < 0.05$) reduction of viability, from 84.1 (SEM 2.0) to 66.6% (SEM 2.7) (Fig. 1). However, adding 250 μM metyrapone to the bilirubin-exposed cells did not alter cell viability.

4. Discussion

Clinical experience has shown that jaundiced infants vary widely in their sensitivity to the neurotoxic effects of bilirubin. Although some variations are assumed to be due to clinical conditions such as immaturity, sepsis, reduced bilirubin–albumin binding, and respiratory failure (hypercarbia), there remain unexplained

differences in vulnerability even between apparently healthy infants with jaundice. Explanations for such differences might emanate from genetic differences which impact on the interaction between bilirubin and the brain. Examples of such factors of interaction may be e.g. bilirubin entry into and clearance from brain, and/or how bilirubin is handled once inside brain cells.

The purpose of this work has been to further study the possible role of an incompletely characterized mitochondrial oxidase as a putative intracellular protective factor against the cytotoxic effect of unconjugated bilirubin in the brain. We have confirmed that the human neuroblastoma cells we have used as a model system do possess the oxidative capacity found in previous studies [13], suggesting that this activity is present in many mammalian cells [9,13]. In mitochondrial membrane preparations from these cells the activity was inhibited by metyrapone, lending further support to the impression that the enzyme in question may belong to the cytochrome oxidases. Recent data suggest that cytochrome P450 2A5 may play an important role in hepatic oxidation of bilirubin [22], but it is not known whether the hepatic and brain metabolic pathways are the same. Since hepatic P450 2A5 is microsomal while the brain enzyme we are studying is mitochondrial, such similarity cannot automatically be assumed.

However, Gazzin et al. [23] have shown distinct, transient up-regulation of P450 1a1, 1a2, and 2a3 in different regions of the brain after exposure to bilirubin. Furthermore, there were regional differences both in magnitude and timing of the responses, with cerebral cortex and superior colliculae showing a much higher and faster response within the first hour, whereas cerebellum and inferior colliculae evinced a much slower and lower response, peaking at about 24 h. These findings seem to support the idea that there is a protective response to an increase in bilirubin levels, through the upregulation of several enzymes. Metyrapone is an inhibitor of the P450 enzymes as well. Thus, assuming the activity of these enzymes is protective, administration of metyrapone should have led to reduced viability in our cell cultures. Our study therefore poses a relevant question to the findings of Gazzin et al.; does the up-regulation of the P450 family of enzymes protect the cell, or is the apparent relationship spurious?

The rate of oxidation of bilirubin in the mitochondrial fractions from our neuroblastoma cells was shown to be almost ten-fold higher than in the fraction prepared from whole rat brain in earlier studies [8]. We believe this may be due to the greater homogeneity of our mitochondrial preparations from one selected cell line compared to the ones obtained from whole animal brains. We have then attempted to inhibit this oxidative action in the living cell with metyrapone to evaluate whether this intervention would increase cell death in cells exposed to levels of bilirubin that in our control experiments led to about 20% reduced viability.

The finding that chemically blocking intracellular bilirubin oxidation did not affect the viability of the cells leads to a series of new questions. Although we have demonstrated the presence of an oxidizing enzyme in mitochondria of neuroblastoma cells, the mechanism appears not to be essential for cell survival in this model. This could be due to other protective properties of the cells, such as active extrusion of unbound bilirubin through channel proteins like p-glycoprotein, which could compensate for and mask the reduced intracellular bilirubin oxidation. One could also speculate that because the specific activity of the enzyme in these cells is much greater than previously shown in preparations from whole brain, perhaps “the margin of safety” is much greater.

Future research should investigate endogenous risk factors, for example mapping differences in the expressions of membrane transporter genes as well as how their expressions may be modulated by exogenous factors, such as disease, drugs, and even bilirubin. To understand the differences in vulnerability to unconjugated hyperbilirubinemia it is important to clarify the interaction between bilirubin and membrane transporters, both at the level of the neuron and at the level of the blood–brain barrier.It should be noted that the CHP-212 cells are cancer cells. Therefore, the lack of positive results in our study may indicate that they are less suited as a model for more vulnerable neonatal neuronal cells.

5. Conclusion

In this study we have shown that adding 250 μM metyrapone to neuroblastoma cells in culture exposed to a bilirubin concentration of 75 μM does not significantly alter cell viability. Metyrapone, however, had a significant inhibitory effect on bilirubin oxidation in isolated mitochondrial membranes from the same type of cells, lending further indirect support to the hypothesis that the enzyme belongs to the cytochrome oxidases.

Our findings add to the discussion on whether an oxidase on the inner mitochondrial membrane may represent a neuroprotective mechanism in the complex clinical setting of neonatal jaundice. Even if we found no effect on viability in our model, further investigation is needed to fully understand its significance. Hence, we still cannot rule out the possible cytoprotective role of this enzymatic activity in unconjugated hyperbilirubinemia affecting the central nervous system.

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