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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.