In vitro Effect of Bilirubin on Antioxidant Activity and Lipid Peroxidation in the Plasma and Erythrocytes of Cord Blood

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An increased content of bilirubin in the blood of newborns exceeding 250-300 μM is accompanied by the risk of the development of a neurotoxic effect [6]. The mechanism of hyperbilirubinemia in newborns and the mechanism of its toxic effect are not completely understood. Even taking into account the toxicity of bilirubin and the high susceptibility of blood cells in the newborn to damaging influences, it remains unclear whether high concentrations of bilirubin can exert a toxic effect on blood cells (erythrocytes, platelets, etc.). The literature available suggests that the damage to erythrocytes in newborns is due to intensification of free radical processes associated with the generation of reactive oxygen forms [3] and reduced activity of some elements of the antioxidant defense system in the erythrocytes [15], which result in an increased susceptibility of the cells to peroxidation processes [12]. The role of bilirubin in such processes remains obscure. In this connection we undertook an *in vitro* investigation of the effect of bilirubin on the antioxidant activity and lipid peroxidation (LPO) in plasma and erythrocytes of cord blood.

MATERIALS AND METHODS

Citrate plasma and erythrocytes of the cord blood of healthy newborns were used for the experiments. Bilirubin (chemically pure grade, Reanal, Hungary, m.w. 584.6 D) was dissolved in 1% Na_2CO_3. This solution (or vehicle) was added to the plasma samples in a volume ratio of 1:4 (plasma:bilirubin). The final concentration of bilirubin ranged from 40 to 400 μM. The plasma was incubated with bilirubin at 37°C for 30 min. Erythrocytes were washed three times with physiological saline and the bilirubin solution was added at the same volume ratio as to the plasma samples. The incubation was performed under the same conditions. After the incubation the erythrocytes were washed three times with physiological saline by centrifuging the suspension at 1500 g. The supernatant was used for the detection of possible hemolysis by measuring the content of hemoglobin with Synthakon kits. In both the erythrocytes and plasma the level of primary (diene conjugates), intermediate (carbonyl), and end products of LPO were assessed as described elsewhere [5]. Total antioxidant activity (AOA) of the samples was determined in a quercetin-containing medium [4]. The erythrocytes were pretreated after Dubinina [2]. The AOA in the plasma was measured in the same way [4] 2 days after the freezing-thawing procedure. The AOA of bilirubin itself was determined analogously by adding the aliquot of the bilirubin solution to the incubation medium instead of the sample. The catalase activity in the erythrocytes was determined spectrophotometrically [7]. On the 2nd day the content of thiobarbituric acid-reactive substances (TBARS) in the plasma [8]

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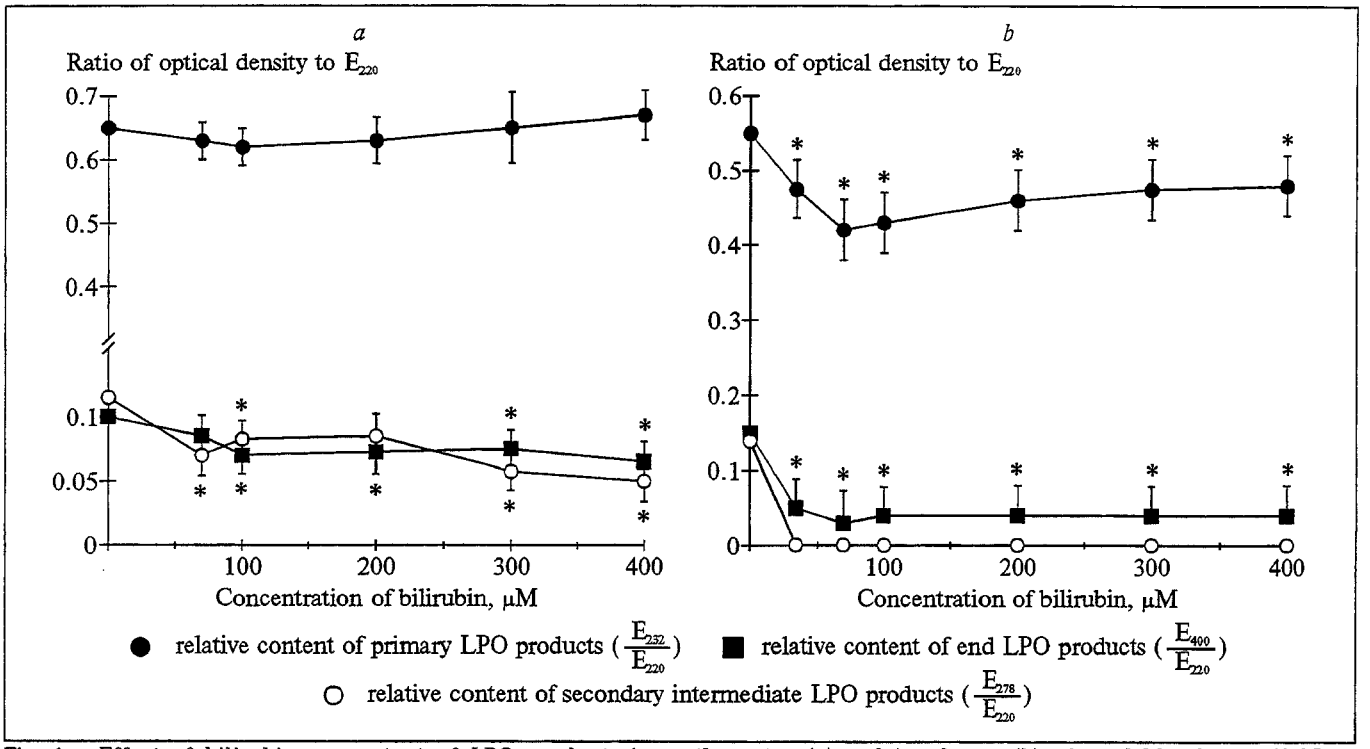

Fig. 1. Effect of bilirubin on content of LPO products in erythrocytes (*a*) and in plasma (*b*) of cord blood. *: $p<0.05$ as compared with the sample without bilirubin.

as well as the activity of ceruloplasmin (CP), an important extracellular antioxidant, were measured. In our experiments we used a preparation of superoxide dismutase (SOD) from erythrocytes and plasma purified according to a method described previously [9].

RESULTS

Bilirubin in the concentration range of 40 to 400 μM did not cause erythrocyte hemolysis, judging from the absence of hemoglobin in the supernatant obtained after the washing procedure. The incubation of cord plasma with bilirubin resulted in a decrease of the LPO products studied: primary products (E_{232}) by 9% and intermediate (E_{278}) by 4-5-fold (Fig. 1). The end products of LPO (E_{400}) were not detected. In the erythrocytes the content of the intermediate and end products of LPO (abs. 278 and 400 nm, respectively) was decreased by approximately 30%, whereas the content of the primary products (abs. 232 nm) was not lowered (Fig. 1). Bilirubin-induced changes in the TBARS content were difficult to assess from our data, since bilirubin itself reacted with TBA, yielding a colored product, so that the contribution of this reaction to the final coloration was difficult to estimate.

Bilirubin affected the AOA in the plasma and erythrocytes in dissimilar ways (Fig. 2). In the plasma AOA progressively decreased for a bilirubin concentration ranging from 100 to 150 μM. Higher doses of bilirubin did not cause a further decrease in AOA. In the erythrocytes AOA rose (from 19 to 31 IU/mg hemoglibin, $p<0.05$) with an increase in bilirubin concentration (Fig. 2). A bilirubin-induced rise of the catalase activity in the erythrocytes was also observed. In the control samples of erythrocytes the catalase activity was $9\pm1\times10^4$ IU/mg hemoglobin, whereas bilirubin in a concentration of 200-400 μM increased the activity of the enzyme to $19\pm2\times10^4$ IU/mg hemoglobin ($p<0.01$). It should be noted that incubation of the plasma with bilirubin did not affect the CP activity at any of the concentrations tested.

The data obtained suggest the inhibition of LPO processes and decrease of AOA in the plasma and erythrocytes of cord blood induced by bilirubin. The LPO inhibition in the plasma and erythrocytes indicates the possible antioxidant effect of bilirubin. According to the literature available, bilirubin is an effective "trap" for peroxyl radicals [7]. The bilirubin-induced inhibition of LPO under our experimental conditions was observed starting from the concentration of 40-60 μM, which also conforms with published data [7]. The effect of bilirubin on the level of LPO in erythrocytes revealed in our experiments is probably mediated through the increase of AOA. There are some data concerning the ability of bilirubin to bind phos-

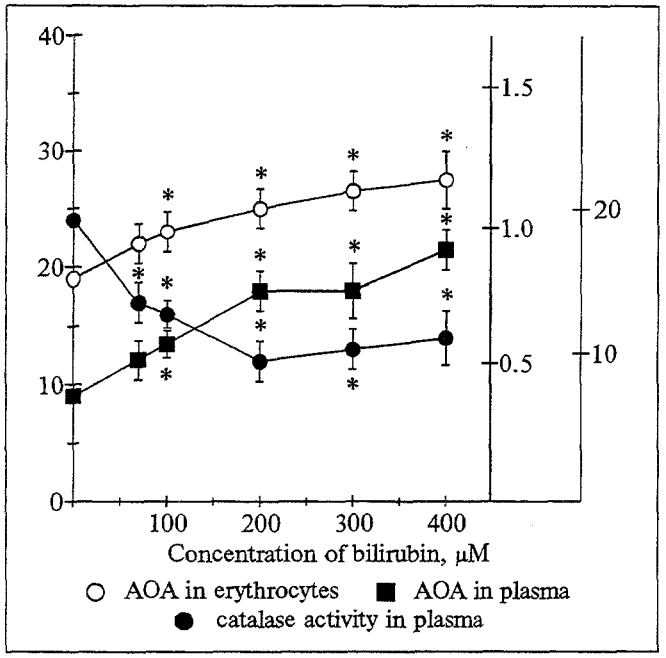

Fig. 2. Effect of bilirubin on AOA and catalase in erythrocytes and plasma of cord blood. Other designations as in Fig. 1.

pholipids [8,10], which may be one of the mechanisms of LPO inhibition. The inhibition of LPO in erythrocytes may reflect an adaptive reaction of the cells to bilirubin action, associated with an alteration of the lipid composition of the erythrocytes. Thus, although bilirubin caused pronounced changes in the AOA and rate of LPO in the erythrocytes, it evidently did not affect the resistance of the erythrocyte membranes.

Unlike the decrease in LPO products in the plasma and erythrocytes, which may be assumed to be due to similar mechanisms, the changes in AOA in the plasma are different from those in the erythrocytes. Bilirubin in the plasma is present in a protein-bound form. Free bilirubin dissolved in Na_2CO_3 exhibited no AOA in the reaction with quercetin in the concentrations studied. However, protein-bound bilirubin is known to possess antioxidant properties [13,14]. The decrease of AOA in the plasma that was observed in our experiments might have resulted from a bilirubin-induced modification of different substances (predominantly proteins) which are crucial for AOA.

In order to find out whether bilirubin may influence the activity of SOD, one of the major plasma antioxidant enzymes, *in vitro* experiments were carried out with the purified enzyme under analogous conditions. It was shown that bilirubin in the concentration range from 40 to 400 μM did not affect the SOD activity in the reaction with quercetin, thus indicating that its effect is not connected with the two specific enzymes, SOD and CP. It remains unclear what disturbances lead to the reduction of AOA. We believe, however, that the reduced AOA in the cord plasma represents an unfavorable factor that lowers the resistance of the blood cells against the peroxidation processes.

The acknowledgment of a possible AOA of bilirubin leaves open the question of the physiological significance of this phenomenon, especially in the case of jaundice of the newborn. In some pathological states, in particular, in hypoxia of newborn, when the activity of certain elements of the antioxidant defense system is decreased, bilirubin evidently is not effective as an antioxidant, so the level of LPO products in the serum of newborns, regardless of the concentration of bilirubin, remains elevated [10]. High concentrations of bilirubin (more then 180-200 μM) may result (due to a reduced AOA in the plasma) in an increased susceptibility of the blood cells to toxic influences.

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