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Bilirubin selectively inhibits cytochrome *c* oxidase activity and induces apoptosis in immature cortical neurons: assessment of the protective effects of glycoursodeoxycholic acid

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Abstract

High levels of unconjugated bilirubin (UCB) may initiate encephalopathy in neonatal life, mainly in pre-mature infants. The molecular mechanisms of this bilirubin-induced neurologic dysfunction (BIND) are not yet clarified and no neuroprotective strategy is currently worldwide accepted. Here, we show that UCB, at conditions mimicking those of hyperbilirubinemic newborns (50 μM UCB in the presence of 100 μM human serum albumin), rapidly (within 1 h) inhibited cytochrome *c* oxidase activity and ascorbate-driven oxygen consumption in 3 days *in vitro* rat cortical neurons. This was accompanied by a bioenergetic and oxidative crisis, and apoptotic cell death, as judged by the collapse of the inner-mitochondrial membrane potential, increased glycolytic activity, superoxide anion radical production, and ATP release, as well as disruption of glutathione redox status. Furthermore, the antioxidant compound glycoursodeoxycholic acid (GUDCA) fully abrogated UCB-induced cytochrome *c* oxidase inhibition and significantly prevented oxidative stress, metabolic alterations, and cell demise. These results suggest that the neurotoxicity associated with neonatal bilirubin-induced encephalopathy occur through a dysregulation of energy metabolism, and supports the notion that GUDCA may be useful in the treatment of BIND.

Keywords: bilirubin neurotoxicity, glycolysis, glycoursodeoxycholic acid, mitochondrial dysfunction, oxidative stress, respiratory chain.

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As a consequence of the short half-life of fetal erythrocytes, and of the limited ability of the neonate to conjugate and excrete bilirubin, newborn infants often show increased levels of serum unconjugated bilirubin (UCB). This condition, known as the *physiologic jaundice*, is usually resolved by the end of the first week of life without treatment (Ostrow *et al.* 2003; Reiser 2004). However, severe hyperbilirubinemia in neonates with pre-maturity and/or systemic illnesses such as hemolytic disease, acidosis, and hypoxemia enhances their risk for bilirubin-induced neurologic dysfunction (BIND) (McDonald *et al.* 1998; Ostrow *et al.* 2004; Shapiro 2005). In fact, *in vitro* experiments revealed that immature neurons have an increased susceptibility to UCB (Falcão *et al.* 2006). Although the molecular mechanisms of BIND remain to be fully clarified, it was shown to involve

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Abbreviations used: 7-AAD, 7-amino-actinomycin D; APC, allophycocyanin; BIND, bilirubin-induced neurologic dysfunction; CoQ_1, coenzyme Q_1; DAPI, 4′-6-diamidino-2-phenylindole; DIV, days *in vitro*; F1,6P_2, fructose-1,6-bisphosphate; F2,6P_2, fructose-2,6-bisphosphate; F6P, fructose-6-phosphate; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GUDCA, glycoursodeoxycholic acid; HBSS, Hanks' balanced salt solution; HSA, human serum albumin; PBS, phosphate-buffered saline; pNA, *p*-nitroaniline; PPP, pentose-phosphate pathway; TMRE, tetramethylrhodamine; UCB, unconjugated bilirubin.

immunostimulation, accumulation of extracellular glutamate, oxidative stress, apoptosis, and loss of cell viability (Brito *et al.* 2008a; Brites *et al.* 2009). This excitotoxic-like situation prompted us to hypothesize whether UCB would exert the death of immature nerve cells through an impairment of energy metabolism.

Many previous studies have examined the effects of UCB on cerebral energy status but the results have been equivocal. In fact, initial reports of an inhibition of respiration and uncoupling of oxidative phosphorylation, observed in brain homogenates or isolated mitochondria, pointed to mitochondrial dysfunction as an important element of UCB toxicity (Day 1954; Ernster and Zetterström 1956; Mustafa *et al.* 1969). The energy depletion was later on corroborated by the reduced rates of glycolysis and decreased ATP levels observed in Gunn rats and in newborn piglets (Katoh-Semba 1976; McCandless and Abel 1980; Hoffman *et al.* 1996; Park *et al.* 2001). However, discrepant findings were reported in other studies that failed to document significant changes in brain glucose metabolism or oxidative phosphorylation (Diamond and Schmid 1967; Brann *et al.* 1987), whereas others demonstrated that hyperbilirubinemia only disturbs brain energy metabolism in the presence of additional factors that disrupt the blood–brain barrier, such as hypoxia or hyperosmolarity (Ives *et al.* 1988, 1989; Wennberg *et al.* 1991). Thus, further studies are needed to clarify the effects of UCB on brain energy and glucose metabolism in nerve cells, particularly in poor differentiated neurons. This would provide a valuable contribute to the current understanding of the neuropathological effects of UCB, as neuronal energy metabolism is determinant for processes as neurotransmitter release, neurite outgrowth, and cell survival (Mattson and Liu 2002), which are impaired by UCB. Moreover, energy hypometabolism is one of the most consistent and earliest abnormalities seen in mild cognitive impairment (Atamna and Frey 2007), which is particularly relevant in pre-mature jaundiced infants.

Recent data indicate that ursodeoxycholic acid and its glycine-conjugated species are able to prevent UCB-induced apoptosis and loss of cell viability, oxidative stress and immunostimulation (Fernandes *et al.* 2007; Brito *et al.* 2008b), and induces a rapid and sustained decrease in plasma UCB concentrations in Gunn rats, the well-established animal model for severe hyperbilirubinemia (Cuperus *et al.* 2009). Thus, we decided to test the neuroprotective effects of glycoursodeoxycholic acid (GUDCA) in our model of immature neurons.

In this study, we show that UCB at concentrations mimicking pathophysiological conditions (Brito *et al.* 2006), rapidly and selectively inhibits the activity of cytochrome *c* oxidase, the terminal component of the mitochondrial respiratory chain, in immature neurons; this led to an impairment in oxygen consumption, inner-mitochondrial membrane potential ($\Delta\psi_m$) collapse, and apoptosis. These phenomena were associated with an increase in cellular oxidized glutathione, production of superoxide anion radical ($O_2^{\bullet-}$), and a decrease in NADPH. Pre-treatment of neurons with GUDCA prior to exposure to UCB prevented inhibition of cytrochrome *c* oxidase activity together with preservation of glutathione and NADPH status. These data indicate that cytochrome *c* oxidase inhibition may be involved in the neurotoxicity associated with BIND and strongly indicate the possible therapeutic potential of GUDCA in the treatment of this disorder.

Materials and methods

Materials

Neurobasal medium, B-27 supplement, Hanks' balanced salt solution-1 (HBSS-1), HBSS without $Ca^{2+}$and Mg^{2+}(HBSS-2), gentamicin (50 mg/mL), tetramethylrhodamine (TMRE), Mito-SOX-Red, and trypsin (0.025%) were acquired from Invitrogen (Carlsbad, CA, USA). Human serum albumin (HSA) (fraction V, fatty acid free), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), ubiquinone-5 (coenzyme Q_1, CoQ_1), 2,2,4-trimethyl-1,3-pentanediol, sulfosalicyclic acid, and 2-vinylpyridine were purchased from Sigma Chemical Co (St Louis, MO, USA). UCB was also from Sigma and purified as previously described (McDonagh 1979). Cytochrome *c* was obtained from Roche Diagnostics (Heidelberg, Germany), and it was reduced with sodium ascorbate (Sigma) just before use and passed through Sephadex G-25 M (PD-10 columns; Amersham Pharmacia Biotech, Uppsala, Sweden) to remove excess ascorbate. GUDCA, as well as caspases 3 and 9 substrates, N-acetyl-Asp-Glu-Val-Asp-Ac-Leu-Glu-His-Asp-*p*-nitroanilide (pNA), and N-acetyl-Leu-Glu-His-Asp-pNA, respectively, were purchased from Calbiochem (Darmstadt, Germany). Other substrates, inhibitors, enzymes, and coenzymes were purchased from Sigma, Roche Diagnostics or Merck (Darmstadt, Germany).

Neurons in primary culture

Animal care followed the European Legislation on Protection of Animals Used for Experimental and Scientific Purposes (EU directive L0065, 22/07/2003). Neurons were isolated from fetuses of 16- to 17-day pregnant Wistar rats, as described previously (Silva *et al.* 2002). The fetuses were collected in HBSS-1, the brain cortex was mechanically fragmented, and the fragments transferred to a 0.025% (w/v) trypsin in HBSS-2 solution and incubated for 15 min at 37°C. After trypsinization, cells were washed twice in HBSS-2 containing 10% (v/v) fetal bovine serum, and resuspended in Neurobasal medium supplemented with 0.5 mM L-glutamine, 25 μM L-glutamic acid, 2% B-27 supplement, and 0.12 mg/mL gentamicin. Finally, cells were seeded on poly-D-lysine coated tissue culture plates at a density of 2.5×10^5 cells/cm^2 and maintained at 37°C in a humidified atmosphere of 5% CO_2. In this work, we used neurons at 3 days *in vitro* (DIV).

Treatment of neurons

Neurons were incubated in Neurobasal medium without (control) or with 50 μM UCB (from a 10 mM stock solution) in the presence of

100 μM HSA (UCB/HSA molar ratio of 0.5) for 1 h at 37°C. Stock UCB solutions were extemporarily prepared in 0.1 M NaOH under the dark and the pH adjusted to 7.4 using 0.1 M HCl. When appropriate, neurons were pre-incubated with GUDCA (50 μM) 1 h prior to UCB addition.

Determination of the mitochondrial respiratory chain complex activities and citrate synthase

Neurons plated on 60 cm^2 Petri dishes were washed with ice-cold phosphate-buffered saline (PBS) and surviving cells were collected by trypsinization, centrifuged and resuspended in 300 μL of 0.1 M potassium phosphate buffer (pH 7.0). Cell suspensions (containing about 7–8 mg of protein/mL) were frozen and thawed thrice to ensure cell lysis. Enzyme activities were determined in the cell lysates using an Uvikon XL spectrophotometer (Secomam, Domont, France). NADH-CoQ_1 reductase (complex I; EC 1.6.99.3) activity was measured as described by Ragan *et al.* (1987). The activity of succinate-cytochrome *c* reductase (complex II-III; EC 1.8.3.1) was determined following the method of King (1967). Cytochome *c* oxidase (complex IV, EC 1.9.3.1) activity was assessed as described by Wharton and Tzagoloff (1967). Citrate synthase (EC 4.1.3.7) activity was assessed as referred by Shepherd and Garland (1969). Protein concentrations were determined by the method of Lowry *et al.* (1951). All enzyme activities were expressed as nanomoles per minute per milligram of protein, except for cytochrome *c* oxidase, which was expressed as the first-order rate constant (*k*) per minute per milligram of protein (Almeida and Bolaños 2001).

Detection of superoxide anion radical ($O_2^{\bullet -}$)

After incubation in 9.6 cm^2 wells, neurons were incubated in PBS containing MitoSox-Red (2 μM) for 30 min, washed with PBS and fluorescence assessed by flow cytometry, using MitoSox-Red method (Invitrogen), as previously described (Mukhopadhyay *et al.* 2007). For the determination of mitochondrial $O2^{\bullet -}$ by flow cytometry, the measurements were carried out using FACScalibur flow cytometer (15 mW argon ion laser tuned at 488 nm, CellQuest software; Becton-Dickinson Biosciences, San Jose, CA, USA) and the data were collected at 585/42 nm (FL2) and 670LP (FL3) channel. In this study, the data were presented in the FL2 channel. Antimycin A at 20 μM was used for 15 min as a mitochondrial superoxide generator.

Determination of oxygen consumption

Oxygen consumption was determined with a Clark-type electrode (Rank Brothers, Cambridge, UK). Briefly, after the incubation period, neurons plated on 60 cm^2 Petri dishes were collected by trypsinization, centrifuged, rinsed once with buffered Hank's solution, and resuspended in 500 μL of Hank's solution (without glucose). Cell suspensions were kept on ice until used for oxygen consumption (within 1 h). The rates of oxygen consumption were calculated from the slopes (monitored for at least 15 min per trace), and expressed as nanomoles of oxygen consumed per minute per 10^6 cells (Almeida *et al.* 1998). 2,2,4-Trimethyl-1,3-pentanediol was used together with ascorbate to assure the reduced form of cytochrome *c*.

$\Delta\psi_m$ measurements

For fluorescence measurements, neurons incubated in 9.6 cm^2 wells were stained as previously described (Almeida *et al.* 1999) with minor modifications. Briefly, neurons were incubated in Hanks' solution containing 1 μg/mL of TMRE for 30 min at 37°C. Excess dye was removed by washing cells twice with buffered Hanks' solution and covered with 1 mL of Hanks' solution. For each Petri dish, four fluorescence microphotographs were taken with an inverted microscope with a fluorescein filter (excitation filter 480–490 nm; emission filter 510–530 nm) and the intensity of fluorescence was quantified using an image analyzer system (NIH Image Program, La Jolla, CA, USA). The representative selected area was always the same for all experimental conditions studied. The fluorescence intensity corresponding to control cells was arbitrarily assigned a value of 100% fluorescence. The 0% $\Delta\psi_m$ value was obtained when cells were loaded with TMRE in the presence of 10 μM FCCP as previously referred (Almeida *et al.* 2001). The monotically decrease of fluorescence with FCCP assures that we are measuring mitochondrial membrane potential and not the plasma membrane potential, where a different pattern of fluorescence would be observed (Farkas *et al.* 1989).

Metabolite determinations

Neurons (about 3×10^7) cultured in 60 cm^2 Petri dishes and treated as abovementioned were rapidly washed with ice-cold PBS, scrapped off with 0.3 M $HClO_4$, and neutralized with 2 M $KHCO_3$ to pH 6.5. The perchlorate precipitate was removed by centrifugation, and fructose-6-phosphate (F6P) and fructose-1,6-bisphosphate ($F1,6P_2$) concentrations were measured in the supernatants as previously described (Almeida *et al.* 2004); intracellular lactate concentrations were determined in the same neutralized extracts as previously mentioned (Gutmann and Wahlefeld 1974).

For the assessment of fructose-2,6-bisphosphate ($F2,6P_2$), cell extracts were lysed in 0.1 M sodium hydroxide and centrifuged (20 000 *g* for 20 min) as previously described (Almeida *et al.* 2004). Briefly, an aliquot of the homogenate was used for protein determination and the remaining sample was heated at 80°C (5 min), centrifuged (20 000 *g* for 20 min) and the resulting supernatant used for the determination of $F2,6P_2$ concentrations (Van Schaftingen *et al.* 1982; Kawaguchi *et al.* 2001).

For intracellular ATP evaluation, neurons were cultured in 2 cm^2 wells and treated as above. At the end of the incubation period, cells were rapidly washed with ice-cold PBS, scrapped off with 2×0.5 mL of 0.3 M $HClO_4$, and neutralized with 0.5 mL of 2 M $KHCO_3$ at pH 6.5. The perchlorate precipitate was removed by centrifugation, and ATP was determined in the supernatants by chemiluminescence using a commercially available kit following the manufacturer's instructions. The released ATP was considered to be that found in the culture medium and the quantification was an adaptation of the method previously described by Silva *et al.* (1997). Briefly, after incubation period, supernatants were collected, placed on ice and exposed to 2 M $HClO_4$. A solution of 2 M $KHCO_3$ was used to restore pH at 6.5. The perchlorate precipitate was removed by centrifugation, and ATP levels were measured fluorimetrically in the protein-free supernatants.

For glutathione measurement, neurons incubated in 9.6 cm^2 wells were washed with ice-cold PBS and immediately collected by scrapping off with 0.5 mL of 1% (w/v) sulfosalicyclic acid. Cell lysates were centrifuged at 13 000 *g* for 5 min at 4°C, and the supernatants used for glutathione determinations on the same day. Total glutathione content [GS_x, i.e. the amount of reduced

glutathione (GSH) plus two times that of oxidized form (GSSG)] and GSSG were measured and calculated as previously described (Dringen and Hamprecht 1996; García-Nogales *et al.* 1999). GS_X and GSSG concentrations were expressed as nanomoles per milligram of protein.

NADPH concentrations were measured accordingly with García-Nogales *et al.* (1999). In brief, neurons incubated in 2 cm^2 wells were washed with ice-cold PBS, and collected in 200 μL of 0.5 M KOH in 50% (v/v) ethanol. Cell lysates were neutralized (pH 7.8) with 200 μL of 0.5 M triethanolamine/0.5 M potassium phosphate and centrifuged at 13 000 *g* for 2 min at 4°C. A 50 μL aliquot of the supernatant was immediately used for NADPH determination (Wulff 1985), with the exception that NADH was oxidized by incubation of the samples with 0.5 mU/μL lactate dehydrogenase and 1 mM pyruvate (Klingerberg 1985).

Assessment of apoptotic cell death by flow cytometry

Allophycocyanin (APC)-conjugated annexin-V and 7-amino-actinomycin D (7-AAD) (Apoptosis Assay Kit; Becton-Dickinson Biosciences) were used to quantitatively determine the percentage of apoptotic cells by flow cytometry. After incubation in 2 cm^2 wells, neurons were stained with annexin-V-APC and 7-AAD, following the manufacturer's instructions, and they were analyzed on a FACScalibur flow cytometer (15 mW argon ion laser tuned at 488 nm, CellQuest software; Becton-Dickinson Biosciences). The annexin V-APC-stained cells that were 7-AAD negative were considered apoptotic (Almeida *et al.* 2004).

Analysis of apoptotic cell death by 4′-6-diamidino-2-phenylindole (DAPI) nuclear staining

Neurons were fixed with 4% (v/v, in PBS) paraformaldehyde for 30 min at 25°C, rinsed with PBS, and incubated with DAPI (30 μM, Sigma). After 10 min, cells were washed thrice with PBS and their nuclei examined under a fluorescence microscope by an author blinded to the test. A total of ~200 cells per condition in three different cultures were quantified, and the results were expressed as the percentage of condensed or fragmented nuclei.

Caspases 3 and 9 activity assays

Activities of caspases 3 and 9 were measured by a colorimetric method (Calbiochem, Darmstadt, Germany). Cells were harvested, washed with ice-cold PBS, and lysed for 30 min on ice in the lysis buffer [50 mM HEPES (pH 7.4); 100 mM NaCl; 0.1% (w/v) cholamidopropyldimethylammonio-1-propanesulfonate; 1 mM dithiothreitol; 0.1 mM EDTA]. The lysate was centrifuged at 10 000 *g* for 10 min at 4°C and the supernatants were collected and stored at −80°C. Protein concentrations were determined as aforementioned. The activity of caspases 3 and 9 was determined in cell lysates by enzymatic cleavage of chromophore *p*NA from the substrate, according to manufacturer's instructions. The proteolytic reaction was carried out in protease assay buffer [50 mM HEPES (pH 7.4); 100 mM NaCl; 0.1% (w/v) cholamidopropyldimethylammonio-1-propanesulfonate; 10 mM dithiothreitol; 0.1 mM EDTA; 10% (v/v) glicerol], containing 2 mM substrate N-acetyl-Asp-Glu-Val-Asp-Ac-Leu-Glu-His-Asp-pNA for caspase 3 and N-acetyl-Leu-Glu-His-Asp-pNA for caspase 9. Following incubation of the reaction mixtures for 2 h at 37°C, the formation of pNA was measured at λ = 405 nm with a reference filter of 620 nm.

Statistical analysis

Measurements from individual cultures were performed in triplicate. The results are expressed as the mean ± SEM values for the number of culture preparations indicated in the legends. Statistical analysis of the results was performed by one-way analysis of variance followed by the least significant difference multiple range test, and $p < 0.05$ was accepted as statistically significant in all cases.

Results

To investigate the possible role of UCB on mitochondrial function in immature nerve cells, 3 DIV cortical neurons were incubated with UCB at conditions mimicking neonatal jaundice (50 μM UCB + 100 μM HSA) that had previously shown to induce oxidative injury and cell death in mature neurons (Brito *et al.* 2008b; Brites *et al.* 2009). Cells were collected 1 h after treatment for the analysis of the activities of the mitochondrial respiratory chain complexes. Activity of NADH-CoQ_1 (Fig. 1a) was unaffected and that of succinate-cytochrome *c* reductase (Fig. 1b), although slightly reduced, did not significantly change after neuronal exposure to UCB. In contrast, cytochrome *c* oxidase activity (Fig. 1c) was inhibited by UCB in approximately 50% ($p < 0.01$). Finally,

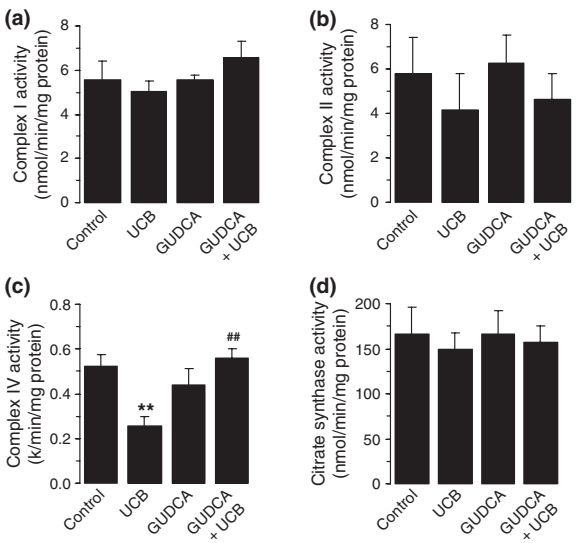

Fig. 1 Unconjugated bilirubin (UCB) selectively impairs cytochrome *c* oxidase activity in immature neurons and glycoursodeoxycholic acid (GUDCA) exerts a preventive effect. Neurons at 3 days *in vitro* were incubated for 1 h with UCB (50 μM) plus human serum albumin (100 μM). When indicated, neurons were pre-treated with GUDCA (50 μM) for 1 h. After incubation, neurons were used for enzyme activity determinations, as indicated in Materials and methods. UCB did not alter NADH-CoQ_1 reductase – Complex I (a) as well as succinate-cytochrome *c* reductase – Complex II-III (b), but inhibited cytochrome *c* oxidase activity that was prevented by GUDCA (c). Additionally, no changes were noticed in citrate synthase activity (d). $^{**}p < 0.01$ versus control; $^{\#\#}p < 0.01$ versus UCB.

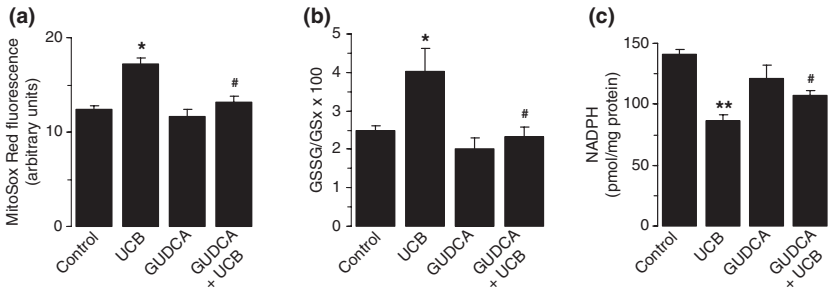

Fig. 2 Unconjugated bilirubin (UCB) produces oxidative stress in immature neurons and glycoursodeoxycholic acid (GUDCA) exerts a preventive effect. Neurons at 3 days *in vitro* were treated as in Fig. 1. After incubation, neurons were used for metabolite assessments as indicated in Materials and methods. UCB-induced oxidative stress through the increase in superoxide anion radical production, as indicated by a higher MitoSox fluoresence intensity (a), an increase in $GSSG/GS_x$ ratio (b), and a decrease in NADPH concentrations (c) that were prevented by GUDCA. $^{**}p < 0.01$ and $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus UCB.

since the activity of citrate synthase (Fig. 1d) was also unchanged, this indicates that no differences in mitochondrial enrichment in our study model account for the decreased complex activity. Since GUDCA has shown to have neuroprotective effects through the prevention of mitochondrial swelling, we have tested the beneficial effects of this bile acid on the UCB-induced alterations on the mitochondrial respiratory chain. GUDCA revealed to be able to completely reverse the inhibition of the cytochrome *c* oxidase activity (Fig. 1c).

Next, we explored the oxidative status of immature neurons exposed to UCB. We observed that UCB markedly induced the production of reactive oxygen species, namely $O_2^{\bullet -}$ (Fig. 2a) and GSSG (Fig. 2b), as well as decreased NADPH concentrations (Fig. 2c). Moreover, pre-incubation of neurons with GUDCA efficiently prevented all these oxidative events caused by UCB.

In view that the mitochondrial respiratory chain thresholds for oxygen consumption (Davey *et al.* 1998), it could be speculated that the level of cytochrome *c* oxidase inhibition caused by UCB might not be enough to impair the mitochondrial function. To elucidate this, we first determined the rate of oxygen consumption in the dissociated neurons previously incubated with UCB. As shown in Fig. 3, UCB significantly reduced the rates of oxygen consumption using glucose, succinate, or ascorbate as substrates, effects that were significantly prevented by GUDCA. The effects on O_2 consumption are related to mitochondria, since both succinate and ascorbate reproduced the same results as with glucose. In addition, in all cases antimycin or potassium cyanide abolished O_2 consumption driven by succinate or ascorbate, respectively (data not shown). The inhibition of oxygen consumption from ascorbate indicates that UCB-

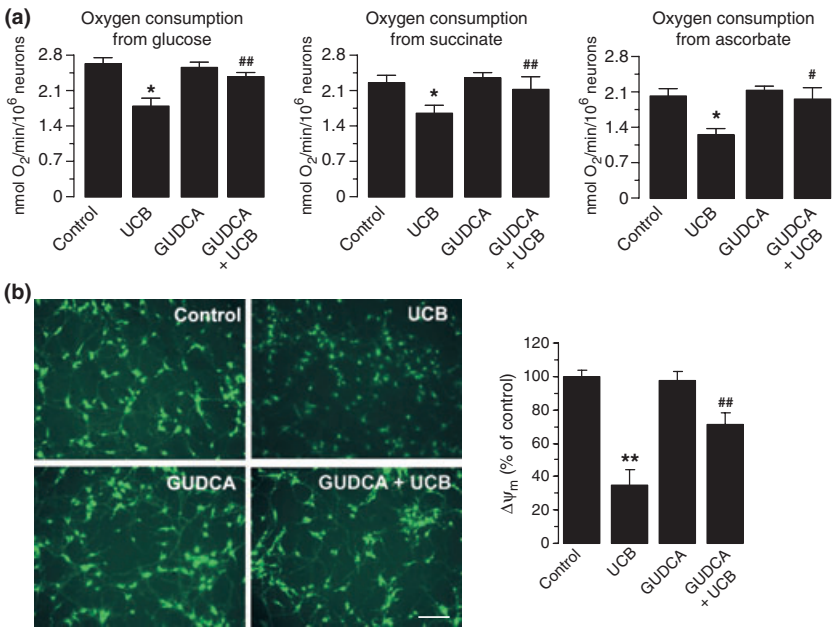

Fig. 3 Unconjugated bilirubin (UCB) impairs cellular oxygen consumption and collapses $\Delta\psi_m$ in immature neurons and glycoursodeoxycholic acid (GUDCA) exerts a preventive effect. Neurons at 3 days *in vitro* were treated as in Fig. 1. After incubation, neurons were either subjected to $\Delta\psi_m$ assessment in the plates or collected for oxygen consumption determinations as indicated in Materials and methods. UCB inhibited the rate of glucose-, succinate- and ascorbate-driven oxygen consumption (a) that was prevented by GUDCA. UCB decreased $\Delta\psi_m$ (b), as assessed by tetramethylrhodamine staining (left panels) and fluorescence quantification (right panel), effects that were prevented by GUDCA. $^{**}p < 0.01$ and $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus UCB (scale bar 50 μm).

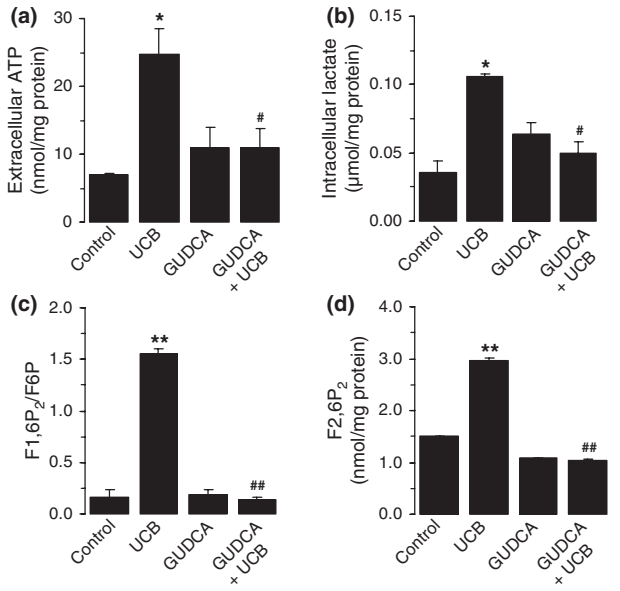

Fig. 4 Unconjugated bilirubin (UCB) increases extracellular ATP content, glycolysis and fructose-2,6-bisphosphate ($F2,6P_2$) levels in immature neurons, and glycoursodeoxycholic acid (GUDCA) exerts a preventive effect. Neurons at 3 days *in vitro* were treated as in Fig. 1. After incubation, extracellular ATP was evaluated, and neurons were lysed for intracellular lactate, fructose-1,6-bisphosphate ($F1,6P_2$), fructose-6-phosphate (F6P) and $F2,6P_2$ measurements, as indicated in Materials and methods. UCB triggered an increase in ATP release (a), intracellular lactate concentration (b), $F1,6P_2$/F6P (c) and $F2,6P_2$ concentration (d), which were prevented by GUDCA. $^{**}p < 0.01$ and $^{*}p < 0.05$ versus control; $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ versus UCB.

inhibition of cytochrome *c* oxidase, affects cell respiration. To further test this possibility, we assessed the $\Delta\psi_m$ as an index of the mitochondrial inner membrane integrity; as depicted in Fig. 3b, UCB caused the collapse of $\Delta\psi_m$, and GUDCA showed ability to restore mitochondrial integrity.

Curiously, although UCB-induced impairment of mitochondrial respiratory chain function was not accompanied by a reduction of the intracellular ATP levels (data not shown), an increase in the concentrations of extracellular ATP (Fig. 4a) was obtained. In addition, it was also observed an increase in the concentration of intracellular lactate (Fig. 4b), as well as in $F1,6P_2$/F6P ratio (Fig. 4c), suggesting an activation of glycolysis, which was further supported by the concomitant elevated levels of $F2,6P_2$ (Fig. 4d). Noticeably, all these effects were abolished by GUDCA.

Finally, we sought to investigate whether the mitochondrial impairment triggered by UCB was associated with neurotoxicity. As shown in Fig. 5a, UCB enhanced the proportion of annexin V^+/7-AAD^- neurons, as assessed by flow cytometry; it also triggered an increase in the proportion of condensed or fragmented nuclei, as visualized with DAPI by fluorescence microscopy (Fig. 5b). Cell death by apoptosis was further corroborated by the increase in the activation of caspase 3 (Fig. 5c). Similar increase in the activation of caspase 9 indicates the involvement of mitochondria in this process. Such effects were again completely counteracted by GUDCA. We can then speculate that the increased glycolytic rate (Fig. 4) is a failed attempt to compensate the mitochondrial impairment. These results support the notion that UCB causes nerve cell death by apoptosis, mainly in immature neurons, and confirms that GUDCA efficiently protects cells against this type of neurotoxicity.

Discussion

Here we show, for the first time, that brief exposure (1 h) of primary cortical immature neurons to UCB in conditions that have relevance to the clinical manifestations of BIND (50 µM UCB + 100 µM HSA) inhibits mitochondrial respiratory chain, at the level of cytochrome *c* oxidase complex.

Mitochondrial dysfunction by UCB appears to involve nitric oxide (NO), accordingly with previous studies (Brito *et al.* 2008b; Mancuso *et al.* 2008) reporting that neuronal oxidative dysruption by UCB is abrogated by the inhibition of neuronal NO synthase. NO is capable of rapidly and reversibly inhibit the mitochondrial respiratory chain and may be implicated in the cytotoxic effects in the CNS (Bolaños *et al.* 1994; Brown and Cooper 1994; Cleeter *et al.* 1994). Additionally, inhibition of the mitochondrial transport chain at the level of complex IV can further produce $O_2^{\bullet-}$ from O_2, a finding also observed in this study (Fig. 2a). Thus, inhibition of mitochondrial cytochrome *c* oxidase by UCB can lead to the formation of both NO and $O_2^{\bullet-}$ and thereby to the formation of peroxynitrite ($ONOO^-$) (Sharpe and Cooper 1998). Interestingly, we previously demonstrated that UCB induces protein oxidation and lipid peroxidation, while diminishes the thiol antioxidant defences, events that were correlated with the extent of cell death, and that GUDCA acts as an antioxidant at protecting neurons against UCB-induced oxidative stress (Brito *et al.* 2008b). This study extended our previous ones by showing that UCB decreases NADPH concentrations (Fig. 2c) in immature neurons, in adition to glutathione oxidation and $O_2^{\bullet-}$ production, confirming oxidative stress by UCB. Moreover, pre-incubation of neurons with GUDCA efficiently prevented these oxidative events, thus highlighting the antioxidant properties of the bile acid in this paradigm. In addition, GUDCA abolished the inhibition of cytochrome *c* oxidase caused by UCB (Fig. 1c), indicating that the mitochondrial respiratory chain damage caused by UCB would be a free radical-mediated process. To the best of our knowledge, this is the first evidence reporting the ability of GUDCA to completely restore the activity of cytochrome *c* oxidase when impaired.

Once a defective respiratory function also includes decreased oxygen consumption, we explored the effects of UCB at such level, since contradictory results were

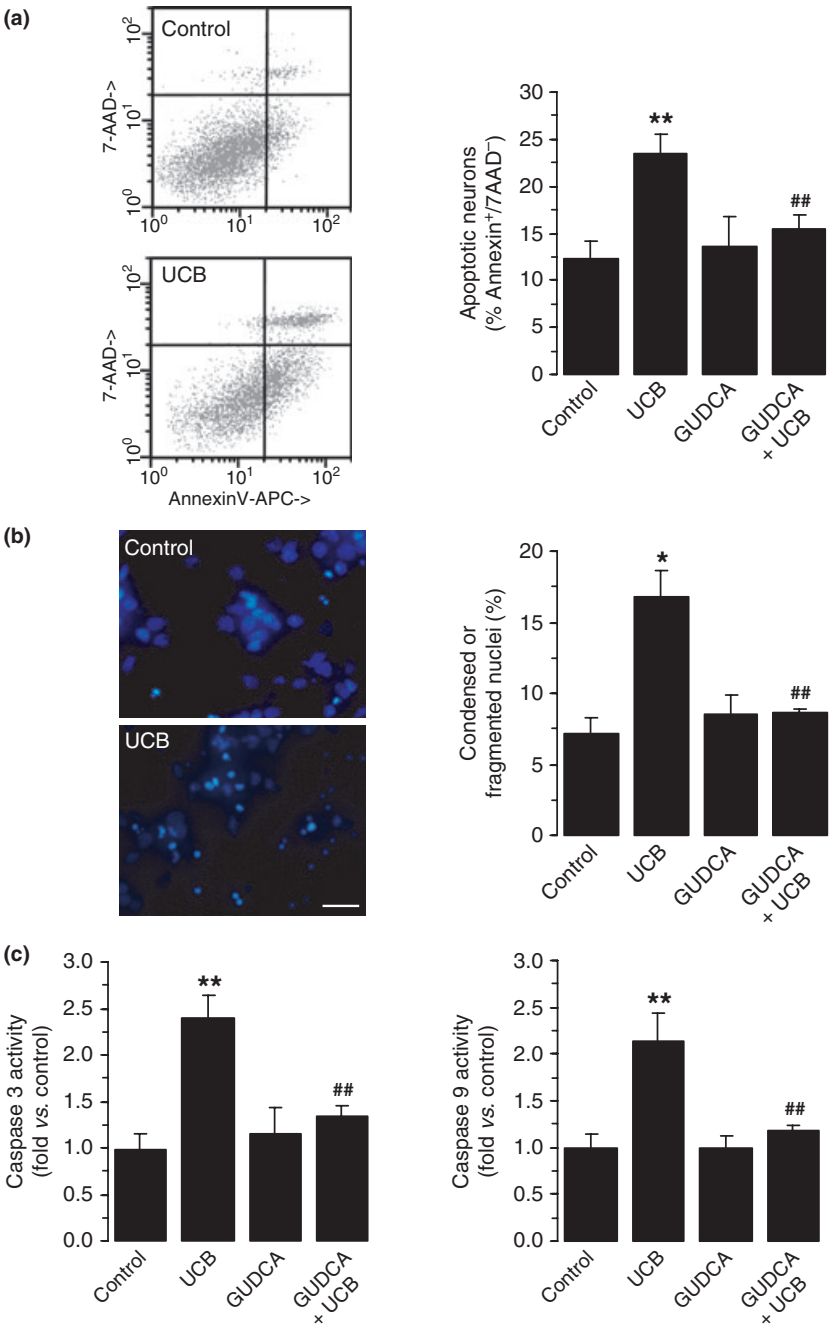

Fig. 5 Unconjugated bilirubin (UCB) triggers apoptotic death in immature neurons and glycoursodeoxycholic acid (GUDCA) exerts a preventive effect. Neurons at 3 days *in vitro* were treated as in Fig. 1. After incubation, neurons were subjected to assessment of apoptotic death by flow cytometry (annexin V^+/7-AAD^-), nuclear condensation or fragmentation in 4′-6-diamidino-2-phenylindole (DAPI)-stained cells, and caspases 3 and 9 activities, as indicated in Materials and methods. UCB increased neuronal apoptosis as measured by the percentage of annexin V^+/7-AAD^- cells (a) (left panel shows a typical diagram; right panel represents the quantification) or fragmented or condensed nuclei (b) (left panel show a typical microphotograph of the DAPI-stained cells; right panel represents the quantification), which was prevented by GUDCA. Data were corroborated by the increase in the activation of caspase 3 and caspase 9 (c) pointing to the involvement of mitochondria. GUDCA was able to markedly prevent this effect. $^{**}p < 0.01$ and $^{*}p < 0.05$ versus control; $^{\#\#}p < 0.01$ versus UCB (scale bar 20 μm).

previously found by several authors not using relevant physiological concentrations or purified UCB (Ernster and Zetterström 1956; Diamond and Schmid 1967; Mustafa *et al.* 1969). Since glucose provides NAD-linked electrons through NADH-CoQ_1 reductase, and succinate FAD-linked ones through succinate dehydrogenase, the inhibition of cell respiration from these substrates by UCB is compatible with, but does not demonstrate, inhibition at the terminal complex, cytochrome *c* oxidase. However, the inhibition of oxygen consumption from ascorbate, which directly supplies electrons to cytochrome *c* oxidase, confirms that the inhibition at the level of this complex by UCB affects cell respiration. This was reinforced by the collapse of $\Delta\psi_m$ produced by UCB indicating decreased mitochondrial membrane potential and mitochondrial dysfunction, which was again prevented when neurons were pre-treated with GUDCA before the exposure to UCB. Permeabilization of the mitochondrial membrane by UCB and increased efflux of cytochrome *c* were previously observed in isolated mitochondria from the brain and liver of adult male Wistar rats

(Rodrigues *et al.* 2000, 2002). Such mitochondrial impairment by UCB was in this study accompanied by an increase in intracellular lactate concentrations and F1,6P_2/F6P ratio, suggesting an activation of glycolysis by stimulation of 6-phosphofructo-1-kinase, i.e., a master regulator of this pathway (Uyeda 1979); this notion was further supported by the observed increased levels of F2,6P_2, i.e., the positive effector of 6-phosphofructo-1-kinase (Van Schaftingen *et al.* 1982). Moreover, intracellular ATP content was unchanged, whereas extracellular levels were increased. Altogether, these results suggest that UCB disrupts the mitochondrial function in immature neurons leading to up-regulation of glycolysis, which reflects the natural metabolic response of cells to cytochrome *c* oxidase deficiency (Bolaños *et al.* 1994; Almeida *et al.* 2004) and may determine the unchanged intracellular ATP levels. In fact, it is conceivable that the up-regulation of glycolysis in these still immature neuronal cells may provide sufficient ATP as a self-protective attempt to support the bioenergetic crisis, as previously demonstrated for astrocytes exposed to injurious stimuli (Bolaños *et al.* 2004). In parallel, the release of ATP by neurons has been suggested to be determined by the production of NO and oxidative stress and to be associated with neuronal cell death by apoptosis, in that ATP is critical to induce several apoptotic events (Figueroa *et al.* 2006; López *et al.* 2006). Noticeably, all these effects were prevented by GUDCA.

Another new finding on the mechanisms of UCB-induced oxidative stress is the decrease of NADPH levels which may contribute to the altered glutathione redox status (Dringen 2000) observed in the presence of UCB. This result is consistent with the recently reported notion that, in neurons, the activation of glycolysis leads to inhibition of the pentose–phosphate pathway, causing glutathione oxidation (Herrero-Mendez *et al.* 2009). Interestingly, through pentose–phosphate pathway, neurons maintain cytochrome *c* in a reduced status to prevent its release and apoptotic death (Vaughn and Deshmukh 2008). In good agreement with this, GUDCA restored GSH levels in immature neurons, as it did with differentiated cells (Brito *et al.* 2008b) and also re-established NADPH values reinforcing its antioxidant capacity. An up-regulation of gamma-glutamyl cysteine synthetase, together with the efficient scavenging of free radicals previously reported for the non-conjugated form of the bile acid (Lapenna *et al.* 2002; Rodriguez-Ortigosa *et al.* 2002; Serviddio *et al.* 2004) shall contribute to the protective actions herewith found.

Unconjugated bilirubin induced mitochondrial dysfunction in 3 DIV neurons by selective inhibition of cytochrome *c* oxidase activity, and decreased mitochondrial membrane potential, but maintained intracellular ATP levels, ultimately leading to apoptotic cell death. The activation of caspase 3, but mostly of caspase 9, points to the activation of the mitochondrial apoptotic pathway in immature neurons after a short exposure to UCB in conditions mimicking an acute neonatal jaundice. To the prevention of this neurotoxicity by GUDCA may account its ability to re-establish the energy metabolism and redox status of the injured cell.

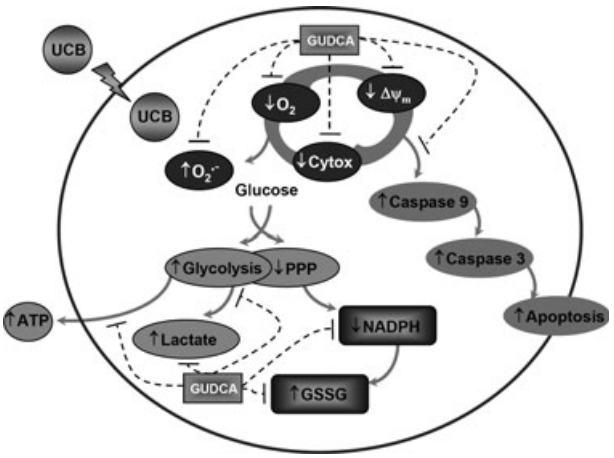

Fig. 6 Schematic representation of the cellular targets involved in unconjugated bilirubin injury to immature cortical neurons and modulation by glycoursodeoxycholic acid (GUDCA). Grey lines with arrowheads indicate the UCB-induced effects, and dashed lines with blocked ends indicate the steps inhibited by GUDCA. UCB interaction with immature neurons causes the selective inhibition of cytochrome *c* oxidase (Cytox) activity, impairs oxygen (O_2) consumption, leads to a loss of mitochondrial membrane potential ($\Delta\psi_m$) and superoxide anion radical production ($O_2^{\bullet-}$) production. UCB also induces the up-regulation of glycolysis and increased amounts of extracellular ATP, together with an inhibition of the pentosephosphate pathway (PPP), as inferred by the decreased NADPH and increased oxidized glutathione (GSSG) levels. All these events shall contribute to the activation of caspases 9 and 3 and apoptotic cell death. GUDCA fully abrogated UCB-induced mitochondrial dysfunction, alterations in energy metabolism and disruption of the redox status, therefore counteracting immature nerve cell demise resulting from UCB exposure.

In conclusion, in this study, we report evidence of UCB induced inhibition of cytochrome *c* oxidase activity in immature rat neurons resulting in respiratory chain dysfunction, a decrease in antioxidant defences and apoptosis, as schematically represented in Fig. 6. The ability of GUDCA to ameliorate UCB-induced mitochondrial respiratory chain dysfunction and restore cellular antioxidant potential supports the efficacy of this compound as a potential treatment for BIND.

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