Bilirubin injury to neurons: Contribution of oxidative stress and rescue by glycoursodeoxycholic acid

Maria A. Brito a,*, Silvia Lima a, Adelaide Fernandes a, Ana S. Falcão a, Rui F.M. Silva a, D. Allan Butterfield b, Dora Brites a

a Centro de Patogénesis Molecular/iMed.UL, Faculdade de Farmácia, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
b Department of Chemistry, Center of Membrane Sciences and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA

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Abstract

It is well established that high levels of unconjugated bilirubin (UCB) can be toxic to the central nervous system, and oxidative stress is emerging as a relevant event in the mechanisms of UCB encephalopathy. In contrast, the hydrophilic bile acid, ursodeoxycholic acid (UDCA), has been reported as a cytoprotective and antioxidant molecule. In this study, we investigated if exposure of rat neurons in primary culture to clinically relevant concentrations of UCB leads to oxidative injury. The contribution of oxidative stress in UCB neurotoxicity was further investigated by examining whether the reduction of NO production by NAME, an inhibitor of nitric oxide synthase, prevents the disruption of the redox status and neuronal damage. Moreover, we evaluated the ability of glycoursodeoxycholic acid (GUDCA), the most relevant conjugated derivative in the serum of patients treated with UDCA, to abrogate the UCB-induced oxidative damage. Cultured rat neurons were incubated with 50 or 100 μM UCB in the presence of 100 μM human serum albumin, alone or in combination with 100 μM NAME or with 50 μM GUDCA, for 4 h at 37 °C. Protein carbonyls, 4-hydroxy-2-nonenal-protein adducts, intracellular glutathione content and cell death were determined. The results obtained showed that UCB induces protein oxidation and lipid peroxidation, while diminishes the thiol antioxidant defences, events that were correlated with the extent of cell death. Moreover, these events were counteracted by NAME and abrogated in the presence of GUDCA. Collectively, this study shows that oxidative stress is one of the pathways associated with neuronal viability impairment by UCB, and that GUDCA significantly prevents such effects from occurring. These findings corroborate the antioxidant properties of the bile acid and point to a new therapeutic approach for UCB-induced neurotoxicity due to oxidative stress.

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1. Introduction

Unconjugated bilirubin (UCB), the end product of heme catabolism, has long been regarded as a waste product, lacking any clear physiologic role. However, there is evidence suggesting that UCB is a potent antioxidant (Barañano et al., 2002; Clark et al., 2000; McDonagh, 1990; Stocker et al., 1987), and that mildly elevated serum UCB levels are associated with a better outcome in diseases involving oxidative stress (Sedlak and Snyder, 2004). Because of the antioxidant properties of UCB, it is nowadays believed that physiologic jaundice of the neonate may have inherent benefits. Nevertheless, in some newborn infants plasma UCB levels can increase dramatically, leading to acute UCB encephalopathy or kernicterus, depending on whether the clinical manifestations of neurological damage are reversible or progress to chronic and permanent clinical sequelae, or even death (American Academy of Pediatrics, 2004; Kaplan and Hammerman, 2005).

A panoply of cellular toxic events occur in different experimental models (Haga et al., 1996; Rodrigues et al., 2002a; Roseth et al., 1998; Seubert et al., 2002; Yeung and Ngai, 2001), reflecting the widespread cytotoxicity resulting from mild to severe hyperbilirubinemia, where impairment of membrane structure, properties and function, as well as disruption of calcium homeostasis appear to play a key role (Brito et al., 2006a,b). Nevertheless, the primary concern with respect to UCB is the potential for neurotoxicity. Our group has been devoted to the study of the neurotoxic effects of UCB and...
has demonstrated that exposure of nerve cells to this molecule, impairs nerve cell functionality, as well as the cytoskeleton assembly, and leads to cell death, either by apoptosis, or necrosis (Falcão et al., 2006; Silva et al., 2001a,b, 2002). We have also shown that UCB induces the extracellular accumulation of glutamate by reducing its uptake (Silva et al., 1999), and/or enhancing its secretion (Falcão et al., 2005; Fernandes et al., 2004; Gordo et al., 2006), which may engender over-stimulation of N-methyl-d-aspartate (NMDA) glutamate receptors and excitotoxicity (Grojean et al., 2000; McDonald et al., 1998). Additionally, we demonstrated that UCB induces an inflammatory reaction, as evidenced by the enhanced production of the pro-inflammatory cytokine tumor necrosis factor (TNF-α) by glial cells and, less markedly, by neurons (Brites et al., in press; Falcão et al., 2005; Fernandes et al., 2004; Gordo et al., 2006). Moreover, we demonstrated that astroglial activation is mediated by TNF-α receptor 1, as well as by activation of mitogen-activated protein kinases (MAPKs) and of the transcription factor nuclear factor-κB (NF-κB) (Fernandes et al., 2006, 2007a). More recently, we provided evidence that exposure to UCB leads to the impairment of neurite development, a fact that may be associated with alterations in the neuronal connectivity during brain development and that may be associated with long-term neurologic dysfunctions (Falcão et al., 2007). Excitotoxicity, compromise of ionic balance, inflammatory reactions, disruption of membrane properties and of cytoskeleton network, as well as impairment of neurite outgrowth and loss of cell viability are usually associated with oxidative stress manifestations (Chinopoulos and Adam-Vizi, 2006; Loh et al., 2006; Mhatre et al., 2004; Neely et al., 1999, 2005; Tyrina et al., 2007; Valencia and Morán, 2004). Therefore, it is conceivable that oxidative stress mediates UCB neurotoxicity, despite the ability of nanomolar concentrations of free UCB to protect neurons against oxidative stress injury (Doré et al., 1999). Nevertheless, it is still poorly characterized the spectrum of oxidative injury to neural cells, as well as its contribution to the mechanisms of neurotoxicity resulting from hyperbilirubinemia.

In oxidative stress conditions, oxidized nucleic acids, proteins and lipids accumulate as a result of the reactive oxygen species (ROS) attack to cell components. Protein carbonyls and 4-hydroxy-2-nenal (HNE), a highly reactive lipid peroxidation decomposition product, are widely used as reliable markers of protein oxidation and lipid peroxidation, respectively (Butterfield and Lauderback, 2002; Uchida, 2003). The antioxidant capacity of tissues can also be assessed by quantification of oxidative stress markers, as glutathione, which reflects the chain-breaking thiol antioxidant capacity (Dringen, 2000). Data accumulated so far indicate that oxidative imbalance, and particularly HNE or HNE-protein adducts, induce complex alterations in a variety of cell functions, therefore playing a key role in the pathogenesis of several diseases of the central nervous system (Brito et al., 2007; Butterfield, 2006; Sultana and Butterfield, 2004), where UCB encephalopathy and kernicterus may eventually be included.

Ursodeoxycholic acid (UDCA), the 7β-hydroxy epimer of Chenodeoxycholic acid, is an endogenous bile acid that has been widely used for the treatment of chronic cholestatic liver diseases since the mid-1980s (Lazaridis et al., 2001) and suggested to have a potential role in the treatment of non-liver diseases associated with increased levels of apoptosis (Rodrigues and Steer, 2001). Following oral administration, it is conjugated in the liver, originating tauursodeoxycholic acid (TUDCA) and, mostly, glycocholic acid (GUDCA) (Rudolph et al., 2002). It was demonstrated that UDCA or its conjugates act as cytoprotective agents, stabilising cell membranes and preventing mitochondrial swelling and cytochrome c release induced by different neurotoxins, thus reducing cellular apoptosis (Güldüttuna et al., 1993; Rodrigues et al., 2000; Silva et al., 2001b; Solá et al., 2002). Our most recent findings showed that GUDCA suppresses the production of the proinflammatory cytokines TNF-α and interleukin (IL)-1β and prevents nerve cell death induced by UCB (Fernandes et al., 2007b). Moreover, it was recently suggested that the cytoprotective mechanism of UDCA, or its conjugates, is mediated by a defense against oxidative stress, pointing to antioxidant properties of the molecule (Lapenna et al., 2002; Perez et al., 2006; Rodrigues et al., 2000; Sastre et al., 2007; Serviddio et al., 2004; Solá et al., 2002).

The present study was undertaken to investigate whether disruption of the redox status occurs upon exposure of primary cultures of rat neurons to clinically relevant concentrations of UCB, and to evaluate the ability of GUDCA to prevent the oxidative injury of neuronal cells induced by UCB. We show that UCB causes protein oxidation, lipid peroxidation and disruption of glutathione metabolism, which course in parallel with the loss of cell viability. These observations, together with the ability of NAME (Nω-nitro-L-arginine methyl ester hydrochloride), an inhibitor of nitric oxide (NO) synthesis, to prevent such effects from occurring suggest that oxidative stress is involved in the pathways of neuronal damage by UCB. Furthermore, we demonstrate that all these events are abrogated in the presence of GUDCA, corroborating the antioxidant properties of this bile acid, and pointing to a new therapeutic approach for neurotoxicity due to UCB-induced oxidative stress.

2. Materials and methods

2.1. Chemicals

Neurobasal medium, B-27 Supplement (50×), Hanks’ balanced salt solution (HBSS-1), Hanks’ balanced salt solution without Ca2+ and Mg2+ (HBSS-2), gentamicin (50 mg/mL) and trypsin (0.25%) were purchased from Invitrogen (Carlsbad, CA, USA). Fetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). Antibiotic antimiycotic solution (20×), human serum albumin (HSA) (fraction V, fatty acid free), glutathione, NAME, alkaline phosphatase-labelled anti-rabbit IgG secondary antibody and Sigma Fast Tablets were from Sigma Chemical Co. (St Louis, MO, USA). UCB was also acquired from Sigma and purified according to the method of McDonagh (1979). GUDCA (minimum 96% pure) was acquired from Calbiochem (Darmstadt, Germany). A
lactate dehydrogenase (LDH) cytotoxicity detection kit was obtained from Roche Molecular Biochemicals (Manheim, Germany). Slot blotting materials including apparatus, nitrocellulose membranes (0.45 μm pore size), and transfer filter papers, as well as the protein assay kit were from Bio-Rad (Hercules, CA, USA). The OxyBlot kit used for protein carbonyl determination was from Chemicon (Temecula, CA, USA) and the HNE antibody used for evaluation of lipid peroxidation was from Alpha Diagnostic International (San Antonio, USA). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Neuron primary cultures

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the institutional animal care and use committee. Every effort was made to minimize the number of animals used and their suffering.

Neurons were isolated from fetuses of 17–18-day pregnant Wistar rats, as previously described (Silva et al., 2002). Cells were morphologically characterized by phase-contrast microscopy and used after 8 days in vitro. Our cell culture protocol greatly avoids contamination by glial cells (Silva et al., 2006), which was lower than 1% as assessed by immunocytochemical staining using primary antibodies raised against glial fibrillary acidic protein of astrocytes.

2.3. Neuron treatment

Neurons were incubated with UCB in the presence of HSA, at UCB to HSA molar ratios of 0.5 and 1.0, which can be attained in the course of a mild or severe neonatal hyperbilirubinemia, respectively. In fact, we have previously found an UCB to HSA molar ratio average value over 0.4 in a group of moderately jaundiced infants (~195 μM UCB) (Brito et al., 1996, 2006a), whereas higher values were reported in more drastic conditions (Ozturk et al., 1996; Perlman et al., 1997). A 10 mM stock solution of UCB was prepared in 0.1N NaOH and used immediately after preparation. Neurons were incubated with 50 or 100 μM UCB, or no addition (control), in the presence of 100 μM HSA, for 4 h at 37°C. The pH value was restored to 7.4 by addition of equal amounts of 0.1N HCl, and all the experiments were performed under light protection (vial wrapped in tin foil and dim light) to avoid photodegradation. In these experimental conditions, we have previously shown that UCB induces neurotoxic effects such as release of glutamate and pro-inflammatory cytokines, as well as activation of NF-κB, without excessive compromise of cell death (Falcão et al., 2006), therefore assuring appropriate conditions to assess the disruption of the redox status by UCB.

To establish the contribution of UCB-induced oxidative injury to neurons, a set of experiments was run where neurons were treated for 30 min with 100 μM NAME, prior to the incubation with UCB (100 μM), performed as described above. A 100 mM stock solution of NAME was prepared in phosphate buffered saline (PBS), pH 7.4. In these conditions, selected from preliminary experiments performed in our laboratory, the loss of cell viability in NAME-treated neurons was insignificant as compared to non-exposed controls, therefore assuring the absence of NAME toxicity to neurons.

In order to investigate the cytoprotective effect of GUDCA, neurons were pre-treated for 1 h with 50 μM GUDCA prior to the addition of 100 μM UCB. In parallel, control experiments were performed where cells were treated with GUDCA alone. GUDCA solution was obtained from a 5 mM stock solution in PBS, pH 7.4. The dose of GUDCA was chosen based on the literature in order to mimic as much as possible the physiological bile acid level present in the serum of patients treated with 450–600 mg/day UDCA (Podda et al., 1990; Simoni et al., 1995), a concentration that have demonstrated no toxicity to neurons, as cell viability was not affected following incubation with the bile acid alone (Silva et al., 2001b).

After treatment, attached cells were collected for evaluation of protein oxidation, lipid peroxidation and glutathione metabolism, while the cell-free medium was used for LDH measurement.

2.4. Assessment of protein oxidation

The formation of protein carbonyls is an indication of oxidative stress and a key marker of protein oxidation (Butterfield and Lauderback, 2002; Butterfield and Stadtman, 1997). Protein-resident carbonyls were measured by slot blot analysis of the 2,4-dinitrophenylhydrazone (DNP-hydrazone) adducts of the carbonyls formed by reaction with 2,4-dinitrophenylhydrazine (DNPH). At the end of the incubation, the medium was removed by aspiration, the cells were rinsed twice with ice-cold PBS and harvested by scraping into 50 μL of ice-cold PBS. The cell suspension was immediately frozen at −80°C until analysis. Just before analysis, samples were thawed on ice and homogenized by sonication. The formation of protein carbonyls was quantified using the OxyBlot kit, according to the manufacturer’s instructions with some modifications, as previously described (Brito et al., 2004; Drake et al., 2002; Kanski et al., 2001). Briefly, a 5 μL aliquot was mixed with an equal volume of 12% SDS and derivatized with 10 μL of DNPH solution. After 20 min, the reaction was stopped by neutralization with 7.5 μL of a 2-M Tris in 30% glycerol solution. After protein determination, a 250 ng protein aliquot of the sample solution was subjected to slot blot analysis and standard immunoblotting techniques were performed using a rabbit-anti-DNP-hydrazone protein adduct polyclonal primary antibody (1:133) and an alkaline phosphatase-labelled anti-rabbit IgG secondary antibody (1:8000). The protein stain was developed by application of Sigma Fast Tablets (BCIP/NBT substrate), and line intensity quantification was achieved using ImageJ software (1.29×, National Institutes of Heath, USA), after scanning into Adobe Photoshop (Adobe Systems Software, Uxbridge, UK).
2.5. Evaluation of lipid peroxidation

Lipid peroxidation, a widespread event in situations of oxidative stress, can be assessed by estimation of HNE, which is considered one of the major reactive products and a specific marker of lipid peroxidation (Butterfield and Lauderback, 2002; Uchida, 2003). Thus, in the present study, levels of protein-bound HNE were determined by slot blot analysis of neurons. At the end of the incubation period, the cells were collected, stored and homogenized as described for protein oxidation. The HNE-protein adducts were detected on the nitrocellulose membrane using a rabbit anti-HNE primary antibody (1:5000) and an alkaline phosphatase-labelled anti-rabbit IgG secondary antibody (1:8000), as previously described (Brito et al., 2004; Lauderback et al., 2002). Sigma Fast Tablets were used as the colorimetric substrate for alkaline phosphatase and the blots were quantified with ImageJ, as referred above.

2.6. Glutathione determinations

The tripeptide glutathione is the most abundant thiol present in mammalian cells, playing an important part in the cellular detoxification of ROS (Dringen, 2000). Total glutathione (GSt) was determined by an enzymatic recycling procedure: the sulphhydryl group of the molecule reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; Ellman’s reagent) producing a yellow colored 5-thio-2-nitrobenzoic acid (TNB), and the disulfide is reduced by NADPH in the presence of glutathione reductase (Griffith, 1980; Tietze, 1969). Following addition of 600 μL ice-cold 5% perchloric acid (six-well culture plates), cells were detached by scraping and transferred to eppendorf tubes. The suspension was sonicated and then neutralized with a 0.76 M KHCO₃ solution. GSH levels were calculated from the absorbance measured at 490 nm, using a PR 2100 microplate reader (Bio-Rad Laboratories, Hemel, Hemel Hempstead, UK). All readings were corrected for the possible interference of UCB absorption and the results expressed as percentage of LDH release, obtained by lysing non-incubated cells with 2.0% Triton X-100 in Neurobasal medium for 30 min.

2.7. Quantification of cell death

Cell death was evaluated by measuring the LDH released by cells with a disrupted membrane, a feature of the overall cell injury (Fink and Cookson, 2005). Enzyme activity was quantified in the incubation medium using the cytotoxicity detection kit, LDH, as previously described (Silva et al., 2002, 2006). The reaction was performed in a 96-well microplate and the absorbance measured at 490 nm, using a PR 2100 microplate reader (Bio-Rad Laboratories, Hemel, Hemel Hempstead, UK). All readings were corrected for the possible interference of UCB absorption and the results expressed as percentage of LDH release, obtained by lysing non-incubated cells with 2.0% Triton X-100 in Neurobasal medium for 30 min.

2.8. Statistical analysis

Results are expressed as means ± S.E.M. from at least four separate experiments performed in duplicate. Differences between groups were determined by one-way ANOVA using Instat 3.05 (GraphPad Software, San Diego, CA). Statistical significance was considered when P values were lower than 0.05.

3. Results

3.1. Unconjugated bilirubin induces protein oxidation in neurons

We have been compiling evidence that oxidative stress is involved in the mechanisms of UCB cytotoxicity. In this regard, we noticed that blood samples from moderately jaundiced neonates present higher levels of membrane-bound hemoglobin (Brito et al., 2006a,b), which is considered an indicator of oxidative stress (Sharma and Premachandra, 1991). Additional studies, based on spin-labeling electron paramagnetic resonance spectroscopy analysis, indicated that UCB disrupts the redox status of isolated mitochondria (Rodrigues et al., 2002a), intact nerve cells (Rodrigues et al., 2002b), and dipalmitoyl phosphatidylcholine liposomes (our unpublished observations). Moreover, injury to neocortical synaptosomes was linked to oxidative stress (Brito et al., 2004). To investigate the participation of protein oxidation in UCB-induced damage to neurons, primary cultures of rat neurons were exposed to UCB, at clinically relevant conditions (UCB to HSA molar ratios of 0.5 and 1.0), for 4 h, and the levels of protein carbonyls were determined by slot blot analysis. As shown in Fig. 1A, UCB caused an increase in the line staining of nitrocellulose membranes, which reflects the formation of protein carbonyls and, thus, the occurrence of protein oxidation in primary cultures of rat neurons. The line staining quantification (Fig. 1B) showed a ~704 and ~972 a.u. raise over control values by UCB-treatment (P < 0.01), indicating that protein oxidation in neuronal cells increase by ~19 and ~27%, respectively, following exposure to the lowest and highest UCB to HSA molar ratio tested in the present study.

3.2. Unconjugated bilirubin induces lipid peroxidation in neurons

To further investigate the influence of UCB on oxidative injury to neurons, the levels of lipid peroxidation were determined after treatment with UCB. Since HNE is a major aldehyde produced during lipid peroxidation, HNE-protein
adducts were measured by slot blot analysis, after incubation of neuronal cells in culture with UCB. As shown in Fig. 2A, UCB incremented the line staining of nitrocellulose membranes, which indicates the formation of HNE-protein adducts and, thus, the occurrence of lipid peroxidation. The ~240 and ~330 a.u. increase \( (P < 0.05) \) observed after treatment with UCB (Fig. 2B) reflects a ~15 and ~20% raise in lipid peroxidation induced by exposure of neurons to UCB, at the lowest and highest concentrations assayed (UCB to HSA molar ratios of 0.5 and 1.0), respectively.

### 3.3. Unconjugated bilirubin impairs glutathione homeostasis in neurons

Having established that UCB promotes oxidative injury to neuronal proteins and lipids, it appeared relevant to have an insight into the cellular anti-oxidant defense mechanisms. To this end we decided to evaluate the levels of glutathione, a low molecular weight thiol with important functions as antioxidant (Dringen, 2000). The concentrations of intracellular glutathione were determined after incubation of rat neurons in primary cultures with UCB, at 50 and 100 \( \mu M \), in the presence of 100 \( \mu M \) HSA (UCB/HSA molar ratios of 0.5 and 1.0, respectively), for 4 h. Treatment of neurons with UCB caused a decrease in the intracellular concentration of GST (2.3–3 nmol/mg protein), which ranged from ~16% to ~20% change from control by treatment with 50 or 100 \( \mu M \) UCB, respectively. Regarding GSH levels (Fig. 3), they corresponded to around 95% of GST in control experiments and followed a similar profile after treatment with UCB. These results point to the disruption of glutathione homeostasis by UCB, where consumption of antioxidant defences takes place, therefore rendering the cells more vulnerable to oxidative damage of cell components.

### 3.4. Neuronal cell death courses in parallel with unconjugated bilirubin-induced oxidative stress

We have previously shown that UCB impairs cell function and induces cell death, based on the evaluation of several indicators of cytotoxicity, as nuclear fragmentation, caspase 3 activation, cytocrome c release, trypan blue dye exclusion, MTT reduction assay, and LDH release (Rodrigues et al., 2000; Silva et al., 2001b, 2002). We have also demonstrated that cell death occurs by both apoptosis and necrosis and that the extent of cell death by both mechanisms is equivalent (Falcão et al., 2006; Fernandes et al., 2004). In this study, we measured the LDH release by cells with a disrupted membrane as a tool to ascertain the extent of cell death and to establish the relationship between oxidative effects and cytotoxicity. As expected based on our previous studies, exposure of neurons to
UCB for 4 h led to a dose-dependent release of LDH (Fig. 4), which raised from 4.8 ± 0.6% in control experiments to 7.7 ± 1.0% and 9.1 ± 1.4% in samples treated with 50 and 100 μM UCB (UCB to HSA molar ratios of 0.5 and 1.0), respectively. It is worthwhile to point out that the UCB-induced cell death was already significant (P < 0.05) at the lowest UCB concentration, a condition that is easily achieved in the course of a moderate hyperbilirubinemia. The parallelism between the neuronal viability impairment and the oxidative lesion of proteins and lipids induced by UCB was attested by the correlation coefficients obtained between both protein carbonyls and HNE-protein adducts levels and LDH release by nonviable cells, which were r = 0.999 and 0.998 (P < 0.05), respectively.

3.5. Neuronal oxidative disruption induced by unconjugated bilirubin is abrogated by inhibition of nitric oxide synthesis

To confirm the relevance of oxidative stress in the pathways of neuronal damage by UCB, additional experiments were performed where cells were pre-treated with NAME, a NOS inhibitor. Thus, after incubation of neurons with 100 μM UCB (UCB/HSA molar ratio of 1.0) for 4 h, in the absence or presence of 100 μM NAME, biomarkers of oxidative stress and of cell viability were evaluated and the results were expressed as % of controls (Table 1). Protein oxidation was selected as a tool to assess membrane oxidative damage, since it showed to be a more accurate and sensitive parameter. Thus, measurement of protein carbonyl levels revealed that the 24% increase over control values observed in UCB-treated samples declined to a 7% elevation in cells pre-treated with NAME. These results indicate that inhibition of the reactive oxygen/nitrogen species production, namely that of NO, leads to a significant reduction of the UCB-induced oxidation of neuronal proteins (P < 0.05 vs. UCB alone). On the other hand, the decrease in GSH levels, which were about 80% of control values in UCB-treated cells, was counteracted by pre-treatment with NAME (P < 0.05 vs. UCB alone), which nearly restored basal levels. Furthermore, the UCB-induced cell death, which corresponded to >80% elevation over control values, was markedly prevented by NAME (P < 0.01 vs. UCB alone). These studies, showing that blockade of the NO production abrogates UCB-induced oxidative injury to neurons, indicate that oxidative stress plays a role in the mechanisms of cell injury by UCB.

3.6. Glycoursodeoxycholic acid prevents unconjugated bilirubin-induced protein oxidation

Previous in vitro and in vivo studies have demonstrated that both UDCA and its taurine derivative, TUDCA, have antioxidant properties, preventing the disruption of the redox status induced by several insults and in different experimental models (Bernardes-Silva et al., 2004; Perez et al., 2006; Rodrigues et al., 2001; Solá et al., 2002). However, as far as we know, there are no reports about the ability of UDCA to prevent oxidative damage in nerve cells, as well as on the antioxidant efficacy of the glycine-conjugated derivative, GUDCA, the prevalent serum bile acid in patients taking UDCA therapy (Lazaridis et al., 2001; Rudolph et al., 2002). Thus, we decided to evaluate whether GUDCA is able to counteract the membrane oxidative damage induced by exposure of neurons to UCB. To this end, after incubation of neurons with 100 μM UCB (UCB/HSA molar ratio of 1.0) for 4 h, pre-exposed or not to 50 μM GUDCA, levels of protein carbonyls were determined. Co-incubation experiments showed that UCB-induced protein oxidation was abrogated by GUDCA (Fig. 5). In fact, the data obtained disclosed that GUDCA is able to counteract the protein carbonyls formation by more than 20% (P < 0.05 when compared with UCB alone) and, thus, to restore control levels.

3.7. Glycoursodeoxycholic acid hinders unconjugated bilirubin-induced depletion of glutathione

UDCA was previously shown to prevent oxidative injury in hepatocytes by increasing the rate of glutathione synthesis and, consequently, the intracellular content of GSH (Mitsuyoshi

![Fig. 4. Unconjugated bilirubin (UCB) induces neuronal cell death. Rat neurons in primary culture were treated without (control) or with 50 or 100 μM UCB, in the presence of 100 μM human serum albumin (HSA), corresponding to UCB to HSA molar ratios of 0, 0.5 and 1.0, respectively, for 4 h at 37 °C. Cell death was evaluated by measuring the activity of lactate dehydrogenase (LDH) released by nonviable cells to the incubation medium using the LDH cytotoxicity detection kit, as described in Section 2.* P < 0.05 and **P < 0.01 vs. control.](image-url)
et al., 1999; Rodriguez-Ortigosa et al., 2002; Serviddio et al., 2004). The ability to restore mitochondrial GSH levels in hepatic cells was also described for TUDCA (Colell et al., 2001), but nothing has been reported concerning GUDCA or non-hepatic cells. Hence, we decided to test whether the presence of GUDCA would prevent the decrease in the intracellular thiol content resulting from UCB interaction with neurons in primary culture. The quantification of GSH levels in cells pre-exposed or not to the bile acid demonstrated the ability of GUDCA to prevent the alterations in glutathione homeostasis originated by UCB (Fig. 6). Indeed, GUDCA-treated cells presented levels of GSH similar to those of controls, reflecting the ability of the bile acid to hinder the 20% decrease of the intracellular thiol originated by UCB (P < 0.05 from UCB alone), and indicating that the GUDCA antioxidant defense against UCB-induced injury is mediated by glutathione.

3.8. Glycoursodeoxycholic acid protects neurons from unconjugated bilirubin-induced cell death

Apart from our own most recent studies showing that GUDCA is able to abrogate astroglial cell death (Fernandes et al., 2007b), there are no reports on the efficacy of this molecule to counteract the loss of cell membrane integrity characteristic of necrosis induced by an injurious stimulus. This prompted us to evaluate the aptitude of GUDCA to prevent LDH release induced by UCB. To this end, rat neurons were pre-treated for 1 h with 50 μM GUDCA prior to the addition of UCB. This resulted in a 1.9-fold elevation of cell death resultant from UCB exposure was greatly counteracted by GUDCA (P < 0.01), which prevented the release of LDH by ~78%. These results demonstrate the ability of GUDCA to protect the demise of neuronal cells due to UCB interaction.

4. Discussion

The present study shows that exposure of primary cultures of rat neurons to clinically relevant concentrations of UCB leads to oxidative damage of cell components, as well as to impairment of the antioxidant defences and cell viability, events that are abrogated by blockade of NO production. These findings, when taken together with our previous studies revealing signs of a disturbance of the redox status (Brito et al., 2004, 2006a;b; Rodrigues et al., 2002a,b), indicate that oxidative stress is involved, at least in part, in UCB-induced...
neurotoxicity. Additionally, we provide evidence that GUDCA is able to abrogate the oxidative injury resulting from UCB interaction with neurons, which indicates a therapeutic potential of this hydrophilic bile acid in conditions where oxidative stress is implicated in neuronal damage. The results obtained in the present study demonstrate that exposure of neurons to UCB leads to protein oxidation and lipid peroxidation, as well as to disruption of glutathione homeostasis. Interestingly, the extent of protein oxidation and lipid peroxidation increases is in the same order of magnitude of those observed in neuronal cells exposed to amyloid-β peptide (1–42) and acrolein (Kanski et al., 2001; Pocernich et al., 2001). It is also worthwhile to point out that the ~20% variation in GSH levels is in the same order of that induced by HNE in Neuro2A cells, at an equivalent incubation period (Neely et al., 2005). Data also highlighted that elevation in protein oxidation and lipid peroxidation is correlated with a loss of cell viability, thus suggesting the involvement of oxidative stress in UCB neurotoxicity. This postulation is further corroborated by the correlation coefficients ($r \geq 0.99$) obtained between the oxidative stress markers evaluated in the present study and both the release of TNF-α and the activation of NF-κB reported in our previous work, performed in the same experimental conditions (Falcão et al., 2006). Still reinforcing the relationship between redox disruption and cell impairment, we have recently observed (Brito et al., 2008) that UCB-induced neuronal oxidative injury is counteracted by increasing the cellular antioxidant machinery through supplementation with the glutathione precursor, N-acetylcysteine, a molecule that not only increases the intracellular thiol stores (Dringen, 2000) but also scavenges ROS (Estany et al., 2007; Pocernich et al., 2001). We further demonstrated that glutathione depletion by buthionine sulfoximine, an inhibitor of glutathione synthesis, increases glial susceptibility to the oxidative effects resulting from UCB. Moreover, it is worthwhile to point out that oxidative damage of macromolecules may result in inefficiency of membrane receptors and transporters, impairment of enzyme activity and cytoskeleton assembly, as well as mutations and aberrant protein synthesis, which obviously have important consequences in cell survival (Brito et al., 2007).

In agreement with our assumption, our previous studies demonstrated that UCB injury to synaptosomal membrane vesicles is linked to a disruption of the redox status (Brito et al., 2004). We also showed that nerve cells exposed to UCB present major membrane perturbations, as detected by spin-labeling electron paramagnetic resonance spectroscopy (EPR) analysis, which were associated with significant oxidative injury to membrane lipids and appeared to mediate UCB-induced cell death (Rodrigues et al., 2002b). In line with our studies, there are reports showing an increased ROS production in hepatoma cells (Oakes and Bend, 2005), and indicating that NO mediates UCB-induced cerebral dysfunction in newborn piglets (Park et al., 2002). The role of NO in the cytotoxicity of UCB was further supported by the observation that inhibition of NO synthase by the use of NAME counteracts the neuronal oxidative disruption observed in the present study, which is in agreement with the improvement of cell viability observed in cultured rat oligodendrocytes (Genc et al., 2003). More recently, a strong correlation between experimental jaundice and oxidative stress in rat brains was documented, supporting the concept that oxidative stress is one of the important mechanisms of jaundice-induced encephalopathy (Chroni et al., 2006).

The results obtained in this work, although providing supportive evidence that oxidative stress is involved in the pathways of neuronal damage by UCB, do not exclude other mechanisms, inasmuch evidence indicate that multiple pathways are involved in UCB neurotoxicity (Brito et al., 2006a). One relevant mechanism of nerve cell injury by UCB appears to be excitotoxicity, as indicated by the extracellular accumulation of glutamate, resulting from impaired uptake and/or increased release (Falcão et al., 2006; Silva et al., 2002), as well as by the activation of the NMDA subtype receptors (Grojean et al., 2000). Interestingly, glutamate neurotoxicity, and thus excitotoxicity, is intimately linked to oxidative stress damage (Facheris et al., 2004). Thus, further studies are needed to clarify whether oxidative injury is a primary event resulting from the direct interaction of UCB with neurons in culture or is secondary to the resultant cell disruption, namely by accumulation of glutamate in the synaptic cleft. It is also worthwhile to highlight that the present findings are not in conflict with the antioxidant role of UCB (Baran˜ano et al., 2002; Clark et al., 2000; McDonagh, 1990; Stocker et al., 1987), considering that UCB behaves like a metabolic “double-edged sword” (Kapitulnik, 2004) counteracting oxidative stress-mediated injury in low concentrations but becoming cytotoxic above a certain threshold (Doré et al., 1999; Mineles et al., 1999).

In contrast with the effects of UCB, cells exposed to GUDCA alone did not exhibit increased levels of LDH release, alterations in glutathione homeostasis or oxidation of cellular components. This glycine-conjugated derivative of UDCA significantly inhibited the disruption of cell membrane integrity as well as the oxidative stress manifestations observed following exposure of neurons to UCB. This cellular protection was expected based on previous reports of cytoprotection and antioxidant properties of UDCA. In fact, studies developed by Utanohara et al. (2005) showed that co-incubation with UDCA for 2–6 h prevented the glycochenodeoxycholic acid-induced release of LDH by rat hepatocytes. In addition, GUDCA was shown to efficiently scavenge free radicals and revert the imbalance between free radicals production and the cell antioxidant defences in several experimental models (Bernardes-Silva et al., 2004; Lapenna et al., 2002; Perez et al., 2006; Rodriguez-Ortigosa et al., 2002; Serviddio et al., 2004).

In agreement with previous studies on the protective effect of UDCA, performed in the liver (Rodriguez-Ortigosa et al., 2002) and placenta (Perez et al., 2006), we verified that pretreatment of neurons with GUDCA prevented the UCB-induced protein oxidation, lipid peroxidation and disruption of glutathione metabolism. Therefore, our present results provide evidence for a mechanism of GUDCA neuroprotective action where cell defence against oxidative insults is implicated and glutathione probably play a key role. Regarding the recovery of
GSH levels as a result of neuronal treatment with GUDCA, it is conceivable that it results from a stimulation of the biosynthetic pathway, as previously observed in hepatocytes (Mitsuyoshi et al., 1999; Rodriguez-Ortigosa et al., 2002; Serviddio et al., 2004).

Our own previous studies, based on spin labeling EPR analysis, demonstrated that UDCA, as well as TUDCA, is able to prevent the disruption of membrane dynamic properties induced by interaction of toxic insults with either isolated mitochondria or whole nerve cells (Rodrigues et al., 2001, 2002a,b; Solá et al., 2002). In fact, the alterations of lipid polarity and of the redox status induced by UCB, hydrophobic bile acids or amyloid-β peptide were abrogated by either the unconjugated form or the taurine conjugated derivative of the bile acid. Accordingly, a stabilization of membrane structure by UDCA or its conjugates was also reported by Gülduit et al. (1993). Thus, it is conceivable that the glycerine-conjugated form of the bile acid, by potentially stabilizing the plasma membrane, inhibits UCB interaction at the cell surface or its diffusion through the membrane, therefore preventing neuronal injury. This assumption is in line with the previously suggested competitive displacement of the hydrophobic bile acid taurodeoxycholate by TUDCA (Sreejayan and von Ritter, 1998). Therefore, the protective role of the bile acid appears to be not entirely a function of its direct antioxidant properties (Lapenna et al., 2002), but to also involve other factors such as the membrane stabilization or displacement of a foreign molecule, such as UCB. In addition, it cannot be ruled out the contribution of the antiapoptotic and anti-inflammatory effects to the cytoprotective mechanisms (Bernardes-Silva et al., 2004; Fernandes et al., 2007b; Perez et al., 2006; Rodrigues and Steer, 2001).

In summary, the present study shows that protein oxidation, lipid peroxidation and impairment of glutathione homeostasis are among the UCB-induced injurious effects to rat neurons in primary culture, thus pointing to oxidative stress as one of the interventional events in UCB neurotoxicity. It is also demonstrated the ability of GUDCA to counteract UCB-induced neuronal damage, by abrogating the alterations in the redox status and preventing cell death. By establishing a new pathway of neuronal stress resulting from UCB exposure, and revealing the potential of GUDCA as a new therapeutic approach for UCB-induced oxidative effects, this study opens new possibilities for the management of moderate to severe neonatal hyperbilirubinemia.

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