Molecular Medicine 6(11): 936–946, 2000

Molecular Medicine

Original Articles

Bilirubin and Amyloid-β Peptide Induce Cytochrome *c* Release Through Mitochondrial Membrane Permeabilization

Cecília M. P. Rodrigues, Susana Solá, Rui Silva, Dora Brites

Centro de Patogénese Molecular, Faculdade de Farmácia, University of Lisbon, Lisbon, Portugal

Accepted July 20, 2000.

Abstract

Background: The pathogenesis of bilirubin encephalopathy and Alzheimer's disease appears to result from accumulation of unconjugated bilirubin (UCB) and amyloid-β (Aβ) peptide, respectively, which may cause apoptosis. Permeabilization of the mitochondrial membrane, with release of intermembrane proteins, has been strongly implicated in cell death. Inhibition of the mitochondrial permeability is one pathway by which ursodeoxycholate (UDC) and tauroursodeoxycholate (TUDC) protect against apoptosis in hepatic and nonhepatic cells. In this study, we further characterize UCB- and Aβ-induced cytotoxicty in isolated neural cells, and investigate membrane perturbation during incubation of isolated mitochondria with both agents. In addition, we evaluate whether the anti-apoptotic drugs UDC and TUDC prevent any changes from occurring.

Materials and Methods: Primary rat neuron and astrocyte cultures were incubated with UCB or Aβ peptide, either alone or in the presence of UDC. Apoptosis was assessed by DNA fragmentation and nuclear morphological changes. Isolated mitochondria were treated with each toxic, either alone or in combination with UDC, TUDC, or cyclosporine A. Mitochondrial swelling was measured spectrophotometrically and cytochrome *c* protein levels determined by Western blot.

Results: Incubation of neural cells with both UCB and Aβ induced apoptosis ($p < 0.01$). Coincubation with UDC reduced apoptosis by >50% ($p < 0.05$). Both toxins caused membrane permeabilization in isolated mitochondria ($p < 0.001$); whereas, pretreatment with UDC was protective ($p < 0.05$). TUDC was even more effective at preventing matrix swelling mediated by Aβ ($p < 0.01$). UDC and TUDC markedly reduced cytochrome *c* release associated with mitochondrial permeabilization induced by UCB and Aβ, respectively ($p < 0.05$). Moreover, cyclosporine A significantly inhibited mitochondrial swelling and cytochrome *c* efflux mediated by UCB ($p < 0.05$).

Conclusion: UCB and Aβ peptide activate the apoptotic machinery in neural cells. Toxicity occurs through a mitochondrial-dependent pathway, which in part involves opening of the permeability transition pore. Furthermore, membrane permeabilization is required for cytochrome *c* release from mitochondria and can be prevented by UDC or TUDC. These data suggest that the mitochondria is a pharmacological target for cytoprotection during unconjugated hyperbilirubinemia and neurodegenerative disorders, and that UDC or TUDC may be potential therapeutic agents.

Address Correspondence and reprint requests to: Cecília M. P. Rodrigues, Ph.D., Centro de Patogénese Molecular, Faculdade de Farmácia, University of Lisbon, Av. Forças Armadas, 1600-083 Lisbon, Portugal.
Phone: 351 21 794 6400; Fax: 351 21 794 6491;
E-mail: cmprodrigues@ff.ul.pt.

Introduction

Apoptosis is a silent form of cell death crucial in the development of a variety of embryonic and adult tissues (1), but when malfunctioning, decreased or increased levels of apoptosis are asso-

ciated with a number of clinical diseases (2,3). Distinctive morphologic and biochemical features of apoptosis include membrane blebbing, progressive condensation of chromatin and cytoplasm, cell shrinkage, and subsequent nuclear fragmentation. These events culminate in the characteristic formation of apoptotic bodies, consisting of nuclear fragments and intact cell organelles surrounded by plasma membrane (4,5). For a long time, the absence of mitochondrial changes was a hallmark of apoptosis, but mitochondria has lately been considered as a central executioner of programmed cell death. In fact, disruption of mitochondrial function appears to be a required factor for apoptosis in many cell types (6–8), in contrast to the cell nucleus and DNA fragmentation (9,10). The permeabilization of the mitochondrial membrane, with subsequent release of soluble intermembrane proteins into the cytosol, has been strongly implicated as a mechanism of cell death (11). A number of different signals, such as calcium and reactive oxygen species, and pro-apoptotic proteins directly act at the mitochondrial membrane, and some of them open the permeability transition pore complex.

Nerve cell injury from unconjugated bilirubin (UCB) has been implicated in brain damage during neonatal hyperbilirubinemia and Crigler-Najjar type I syndrome (12,13). In fact, numerous crucial aspects of cell function are impaired by bilirubin (14–21), and our results suggest that UCB toxicity in cultured astrocytes and neurons may be attributed to apoptotic processes (22,23). Though the primary target of bilirubin toxicity is still not known, mitochondria have long ago been described as particularly vulnerable to this toxic stimulus (24–26). However, the underlying mechanism of toxicity is still far from clear. In addition, the major pathological features of Alzheimer's disease include amyloid plaques composed primarily of the amyloid-β (Aβ) peptide, degenerating neurons and neurofibrillary tangles, as well as the presence of numerous activated astrocytes and microglia (27). Neuronal cell death was shown to be directly triggered by fragments of Aβ (28–30) and, high density of apoptotic cells have been found in brain tissue from patients with Alzheimer's disease (31). Although extensive genetic data implicate Aβ in the neurodegenerative cascade of Alzheimer's disease and a key role for this peptide has been strengthened by several findings, the molecular mechanisms underlying its effects on neuronal cell death are still a matter of debate (32). In fact, it remains a central controversy as to whether the disease is a direct effect of the aggregated peptide or due to the induction of secondary events, such as inflammation or free radical generation.

Recent data suggest that the bile salt ursodeoxycholate (UDC) and its taurine-conjugated form, tauroursodeoxycholate (TUDC), increase the apoptotic threshold in both hepatocytes and nonliver cells from agents acting through different apoptotic pathways. Although the precise molecular mechanism of cytoprotection by UDC is unclear, our results indicate that inhibition of the mitochondrial permeability transition is at least one pathway by which UDC protects against apoptosis (33,34). In addition, we recently showed that TUDC prevented apoptosis induced by the neurotoxic 3-nitropropionic acid in neuronal cells through a mitochondrial-dependent pathway that did not involve the permeability transition pore (35). These findings suggest that the mitochondrial membrane may constitute a pharmacological target for UDC and TUDC, where these molecules act as general regulators of cell survival.

The purpose of this study was to: (I) further characterize UCB- and Aβ-induced cytotoxicty in isolated neuronal and glial cells; (II) investigate mitochondrial membrane perturbation during incubation of isolated mitochondria with both toxic stimuli; and (III) evaluate whether the anti-apoptotic agents UDC and TUCD prevent these changes from occurring. Data obtained confirmed that both UCB and Aβ peptide induce significant apoptosis in neural cells. In addition, the results revealed that UCB and Aβ peptide directly induced mitochondrial permeabilization and cytochrome *c* redistribution, suggesting that mitochondrial membrane perturbation is a key event during apoptosis induced by both toxic stimuli. Finally, UDC and TUDC, shown to prevent cell death induced by several apoptotic agents by modulating mitochondrial alterations, also inhibited UCB- and Aβ-induced toxicity in neural cells, as well as in isolated mitochondria. Thus, mitochondria appear to mediate apoptosis induced by UCB and Aβ peptide.

Materials and Methods

Isolation of Rat Primary Neurons and Astrocytes and Cell Culture

Rat neurons were isolated from fetuses of 17-18-day-pregnant Wistar rats as previously described (36) with minor modifications. In

short, pregnant rats were ether anesthetized and decapitated. The fetuses were collected in Hank's balanced salt solution (HBSS-1; Life Technologies Inc., Grand Island, NY) and rapidly decapitated. After removal of meninges and white matter, the brain cortex was collected in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-2). Brain cortexes were mechanically fragmented, transferred to a 0.025% trypsin in HBSS-2 solution, and incubated for 15 min at 37°C. Following trypsinization, cells were washed twice in HBSS-2 containing 10% fetal calf serum, and resuspended in Neurobasal medium (Life Technologies Inc.) supplemented with 0.5 mM L-glutamine, 25 μM L-glutamic acid, 2% B-27 Supplement (Life Technologies Inc.), and 12 mg/mL gentamicin. Aliquots of 1×10^5 cells/cm^2 were plated on 12-well tissue culture plates (Corning Costar Corp., Cambridge, MA) pre-coated with poly-D-lysine, and maintained at 37°C in a humidified atmosphere of 5% CO_2. Every three days, 0.5 ml of old medium was removed by aspiration and replaced by the same volume of fresh medium without glutamic acid. Cells were used after 8 days in culture. Neurons were morphologically characterized by phase contrast microscopy, and by indirect immunocytochemistry for neurofilaments.

Astrocytes were isolated from 2-day-old Wistar rats as described previously (37), with minor modifications. Briefly, the rat brain was collected after decapitation in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc.) containing 11 mM sodium bicarbonate, 71 mM glucose and 1% antibiotic and antimicotic solution (Sigma Chemical Co., St. Louis, MO). Meninges, blood vessels and white matter were then removed. The cortex was homogenized by mechanical fragmentation, and the cell suspension passed sequentially through steel screens of 230, 104, and 73.3 μm pore size. Cells were collected by centrifugation at 700X g for 10 min and resuspended in DMEM supplemented with 10% fetal calf serum (Life Technologies Inc.). Finally, 2.0×10^5 cells/cm^2 were platted on 12-well tissue culture plates (Corning Costar Corp.) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Culture medium was replaced at day 7, and cells were used after 10 days in culture. Astrocytes were morphologically characterized by phase contrast microscopy, and by indirect immunocytochemistry for glial fibrillary acidic protein (GFAP) using a primary rabbit anti-GFAP antibody followed by a fluorescent-labeled secondary goat anti-rabbit antibody.

Induction of Apoptosis

UCB (Sigma Chemical Co.) was purified (38) and dissolved in 0.1 N NaOH to prepare a stock solution at a concentration of 8.6 mM. The stock solution was added to culture medium (without fetal calf serum), containing human serum albumin (Sigma Chemical Co.) to obtain a final UCB concentration of 86 μM, at a 3:1 bilirubin:albumin molar ratio. Although rarely found in jaundiced newborns, this molar ratio was selected to achieve, in short incubation periods, significant interaction of UCB with cells, mimicking the toxic conditions of a prolonged severe hyperbilirubinemia. A concentrated solution of 5 mM Aβ peptide fragment 25–35 (peptide content 84%; Sigma Chemical Co.) was prepared in water. Isolated rat neurons and astrocytes were cultured as described above and then incubated with either 86 μM UCB for 4 hr, or 25 μM Aβ (25–35) for 24 hr, with or without 100 μM UDC (Sigma Chemical Co.). In the combination groups, cells were pretreated with UDC alone for 1 hr prior to incubation with UCB or Aβ peptide. In all studies, the medium was gently removed at the indicated times and scored for nonviable cells by trypan blue dye exclusion. Attached cells were fixed with 4% formaldehyde in phosphate buffer saline (PBS), pH 7.4, for 10 min at room temperature for both detection of DNA fragmentation and morphologic evaluation of apoptotic changes.

Detection of DNA Fragmentation and Morphological Evaluation of Apoptosis

DNA fragmentation was assessed on fixed cells by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay. The specific technique employed uses terminal deoxynucleotidyl transferase to attach fluorescein labeled nucleotides to free 3′-OH DNA ends. Astrocyte permeabilization was achieved using 0.1% Triton X-100 (Boehringer Mannheim GmbH, Mannheim, Germany) in 0.1% sodium citrate, for 5 min at 4°C; whereas 5% Triton X-100 was needed to permeabilize neurones. After rinsing with PBS, cells were processed according to the manufacturer's instructions (Boehringer Mannheim GmbH). In short, incorporated fluorescein was

detected by anti-fluorescein antibody conjugated with alkaline phosphatase. After substrate reaction using Fast Red tablets (Boehringer Mannheim GmbH), stained cells were analyzed under light microscope and photographs were taken with Kodak GOLD ultra400 films (Eastman Kodak Co., Rochester, NY). The number of TUNEL positive cells (i.e., red nuclei) were counted in at least three random microscopic fields for each sample and the mean values expressed as the percentage of apoptotic nuclei.

Morphology was performed as described previously (39). Briefly, fixed cells were incubated with Hoechst dye 33258 (Sigma Chemical Co.) at 5 μg/ml in PBS for 3 min, washed with PBS and mounted with PBS:glycerol (3:1, volume per volume; v/v). Fluorescence was visualized using a Axioskop fluorescence microscope (ZEISS, Germany). Fluorescent nuclei were scored by different people and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei were identified as non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample of approximately 500 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei.

Isolation of Mitochondria

Low calcium brain and liver mitochondria were isolated from adult male (250–350 g) Wistar rats as previously described (40,41). In short, animals were sacrificed by exsanguination under ether anesthesia and brains and livers removed and rinsed in ice-cold homogenate buffer containing 70 mM sucrose, 220 mM mannitol, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N^1,N^1-tetraacetic acid (EGTA) and 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4. Approximately 10 g of minced brain or liver was prepared as a 10% (weight per volume; w/v) homogenate in an ice-cold solution of homogenate buffer using six complete up and down strokes with a speed controlled mechanical skill drill and a teflon pestle. The homogenate was centrifuged at 600X *g* for 10 min at 4°C using an angle-rotor NR 12156 in a Sigma 3K 30 (B. Braun-Biotech Inc., Allentown, PA), and the post-nuclear supernatant was centrifuged at 7,000X *g* for 10 min at 4°C using an angle-rotor NR 12158. The crude mitochondrial pellet was further purified by sucrose-Percoll gradient centrifugation (42). The pellet was resuspended in 2 ml of homogenate buffer, and 1 ml of the suspension was carefully layered onto a 35 ml self-generating gradient containing 0.25 M sucrose, 1 mM EGTA, and Percoll (Sigma Chemical Co.; 75:25, v/v). The mitochondria were purified by centrifugation at 43,000X *g* for 30 min at 4°C. The clear supernatant solution was removed and the lower turbid layer was resuspended in 30 ml of wash buffer containing 0.1 M KCl, 5 mM 3-(N-morpholino)-propane sulfonic acid (MOPS), and 1 mM EGTA, at pH 7.4 and centrifuged at 7,000X *g* for 10 min at 4°C. The resulting mitochondria pellet was washed twice in wash buffer. A final wash was carried out in chelex-100-treated buffer (Bio-Rad Laboratories, Richmond, VA, 200-400 mesh, potassium form) without EGTA. The pellet was resuspended in 4 ml of chelex-100-treated suspension buffer containing 125 mM sucrose, 50 mM KCl, 5 mM HEPES, and 2 mM KH_2PO_4. The usual yield of mitochondria was approximately 25 mg of protein per gram of liver tissue, which is higher than that obtained in preliminary experiments using brain tissue. Mitochondria were used for experiments within 3 hr of isolation. Aliquots were removed for examining the purity of the mitochondria preparation as previously described (43).

Incubation of Isolated Mitochondria with Inducers of Apoptosis and Measurement of Mitochondrial Membrane Permeability

The mitochondrial membrane permeabilization was assessed using a spectrophotometric assay measuring high amplitude rapid swelling of mitochondria as previously described (41,43) with minor modifications. An increase in mitochondrial swelling results in a decrease in optical density. In brief, mitochondria (0.1 mg protein) were incubated in 1 ml of chelex-100-treated respiration buffer (10 mM HEPES, 10 mM succinate, 215 mM mannitol, 71 mM sucrose, pH 7.4) for 10 min at 25°C and swelling was monitored at 540 nm in a Unicam Spectrometer UV2 (Unicam, Portsmouth, NH). Basal values of mitochondria absorbance were measured for 5 min, and the optical density was monitored for an additional 5 min after addition of either 4.3 μM UCB or 125 μM Aβ fragment 25–35. For the coincubation studies, mitochondria were pretreated with either 500 μM UDC,

500 μM TUDC, or 5 μM cyclosporine A (Sigma Chemical Co.), for 5 min at 25°C.

Measurement of Cytochrome c Levels in Supernatants and Mitochondrial Pellets After the Mitochondrial Permeability Assay

Following the permeability assay, mitochondria were spun down at 12,000X *g* for 3 min at 4°C. Aliquots of the supernatant and pellet were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for detection of cytochrome *c* release. Following separation on a 15% electrophoresis gel, proteins were electrophoretically transferred onto nitrocellulose membranes and the immunoblots incubated with 15% H_2O_2 for 15 min at room temperature. Membranes were then sequentially incubated with 5% milk blocking solution, primary monoclonal antibody to cytochrome *c* (PharMingen, San Diego, CA) at a dilution of 1:5,000 overnight at 4°C, and finally, with secondary goat anti-mouse immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) for 2 hr at room temperature. The membranes were processed for cytochrome *c* detection using the enhanced chemiluminescent light (ECL) system from Amersham (Amersham Life Science, Inc., Arlington Heights, IL).

Densitometry and Statistical Analysis

Densitometry was accomplished using a PC coupled to a PRIMAX 9600 Profi VM6575 scanner (Primax International B.V., Utrecht, The Netherlands). Quantitation of the autoradiograms used the ImageMaster 1D Elite densitometric analysis program (Amersham Pharmacia Biotech, Uppsala, Sweden). The fold change in protein levels was calculated based on the corresponding controls. All data are expressed as mean ± standard error of the mean (SEM) from at least three separate experiments. Differences between groups were compared using the unpaired two-tailed Student's *t*-test performed on the basis of equal or unequal variance as appropriate. *P* values lower than 0.05 were considered statistically significant.

Results

UDC Inhibits Apoptosis Induced by UCB and Aβ Peptide

Cell culture studies confirmed that significant apoptotic changes occurred in primary rat neurons and astrocytes after incubation with UCB and Aβ peptide. Fixed neurons were assayed for the characteristic fragmented DNA of apoptosis using nucleotide-labeling (Fig. 1). Following incubation of neurons with UCB, the TUNEL assay showed that 23% of the cells exhibited positive nuclear staining for fragmented DNA, which represented a 3-fold increase from control values ($p < 0.01$). Using bilirubin:albumin molar ratios of 1:1, we were still able to detect significant levels of apoptosis in neurons, almost reaching 15% of apoptotic cells ($p < 0.01$). In addition, 24% of the neurons were apoptotic after treatment with Aβ peptide. Incubation with UDC produced no changes in levels of apoptosis, compared with controls. Moreover, when the toxic agents and UDC were combined

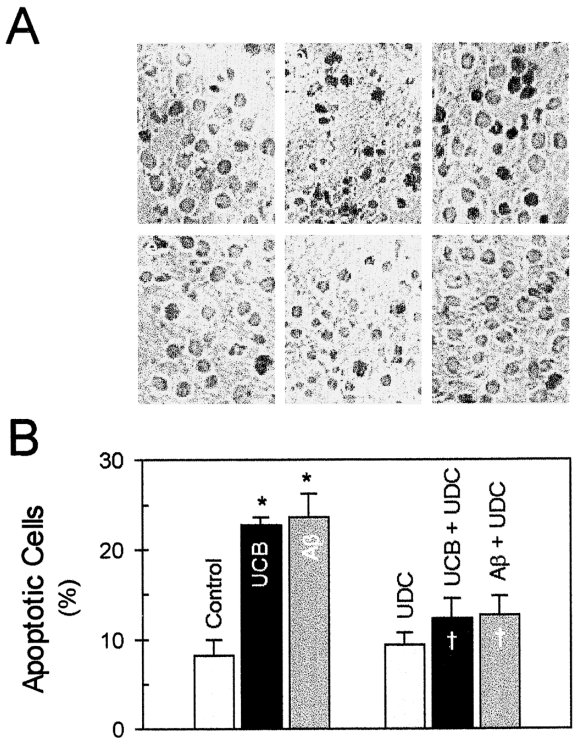

Fig. 1. Apoptosis in primary rat neurons incubated with UCB, and Aβ peptide. Isolated neurons were cultured for 8 days prior to incubation with either 86 μM unconjugated bilirubin (UCB), 25 μM amyloid-β (Aβ) peptide (25–35), a combination of toxic agent plus 100 μM ursodeoxycholate (UDC), or no addition (control). Cells were fixed, and then assayed for nucleotide-labeled genomic DNA as described in "Materials and Methods." (A) TUNEL-positive neurons (dark stain) in cells incubated with no addition (a; control), UCB (b), Aβ peptide (c), UDC (d), UCB plus UDC (e), and Aβ peptide plus UDC (f). (B) Percentage of TUNEL-positive neurons. Values are means ± standard error of the mean (SEM) of at least three separate experiments. *$p < 0.01$ from control; $^{\dagger}p < 0.05$ from toxic stimuli alone.

in the culture medium, the hydrophilic bile salt significantly inhibited neuronal death by apoptosis associated with UCB or Aβ peptide alone ($p < 0.05$). Glial cells were less sensitive than neurons to UCB- and Aβ-induced DNA fragmentation, but UDC protective effect was similar. In fact, primary rat astrocytes incubated with UCB and Aβ peptide exhibited 18 and 16% of apoptosis, respectively ($p < 0.01$). Again, UDC reduced the apoptotic response by >50% ($p < 0.05$). Similar results were also observed when apoptosis was assessed by changes in nuclear morphology after Hoechst staining (Fig. 2). Cell shrinkage, condensation of chromatin, and nuclear fragmentation with formation of apoptotic bodies was evident after incubation of astrocytes with both UCB and Aβ peptide. Moreover, using Hoechst staining, co-incubation with UDC resulted in similar protection of apoptosis induced by both toxic agents.

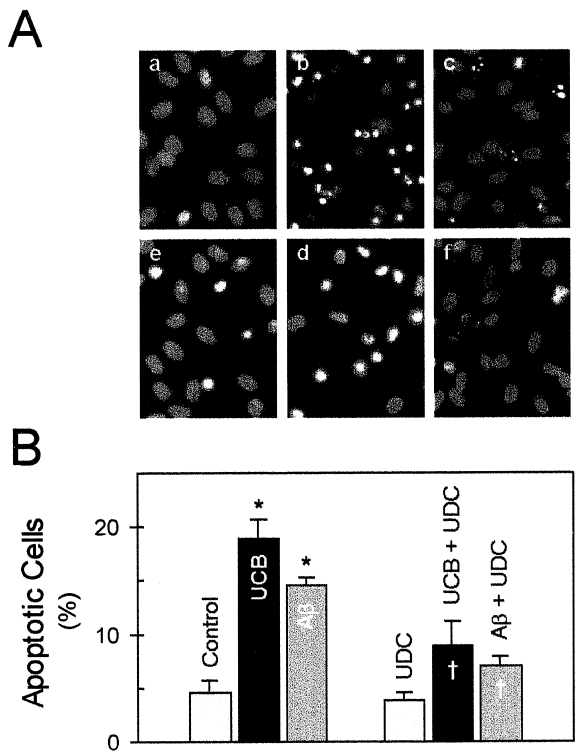

Fig. 2. Apoptosis in primary rat astrocytes incubated with UCB, and Aβ peptide. Isolated astrocytes were cultured for 10 days prior to incubation with either 86 μM uconjugated bilirubin (UCB), 25 μM amyloid-β (Aβ) peptide (25–35), a combination of toxic agent plus 100 μM ursodeoxycholate (UDC), or no addition (control). Cells were fixed, and then assessed for nuclear morphological alterations characterized by condensed chromatin, fragmentation, and formation of apoptotic bodies as described in "Materials and Methods." (A) Fluorescence micrsoscopy of Hoechst staining after incubation of rat astrocytes with no addition (a; control), UCB (b), Aβ peptide (c), UDC (d), UCB plus UDC (e), and Aβ peptide plus UDC (f). (B) Percentage of altered nuclei. Values are means ± standard error of the mean (SEM) of at least three separate experiments. *$p < 0.01$ from control; †$p < 0.05$ from toxic stimuli alone.

UCB and Aβ Peptide Induce Membrane Permeabilization in Isolated Mitochondria

The disruption of mitochondrial function appears to be a key event in apoptosis induced by several stimuli and may mark the commitment to the apoptotic death process (7). Thus, to characterize the effect of UCB and Aβ peptide on these organelles, we assayed the membrane permeability in mitochondria isolated from adult rats. Mitochondria were isolated from rat liver with better yield and purity than from rat brain (data not shown). Since results from preliminary studies using liver and brain mitochondria showed similar trends, we decided to use liver organelles for the remainder of the experiments. Incubations with UCB resulted in high amplitude mitochondrial swelling, which was rapid and dose-dependent. The absorbance of the mitochondrial suspension at 5 min after adding UCB decreased 11% from control with 0.9 μM ($p < 0.05$) and 48% with 8.6 μM ($p < 0.001$); (Fig. 3). The effect of different dosage of Aβ peptide on the mitochondrial membrane permeability was examined and is also shown in Figure 3. Incubations with Aβ peptide resulted in significant decreases in mitochondrial absorbance, which was already evident with concentrations as low as 63 μM Aβ (14%, $p < 0.01$), and continued to increase using 125 μM concentrations (19%, $p < 0.01$).

Since UDC and TUDC previously were shown to exert their protective action at the mitochondrial level using other models, we then investigated the effect of UDC and TUDC on the mitochondrial membrane permeability induced by 4.3 μM UCB and 125 μM Aβ peptide (Fig. 4). Mitochondrial swelling increased 22- and 12-fold over control values after a 5-min incubation with UCB and Aβ, respectively ($p < 0.001$). UDC alone slightly increased swelling ($p < 0.05$); whereas, TUDC produced no significant changes in organelle permeability relative to control. Moreover, during coincubation studies, UDC protected against UCB- and Aβ-induced mitochondrial swelling by 79% ($p < 0.001$) and 50% ($p < 0.05$), respectively.

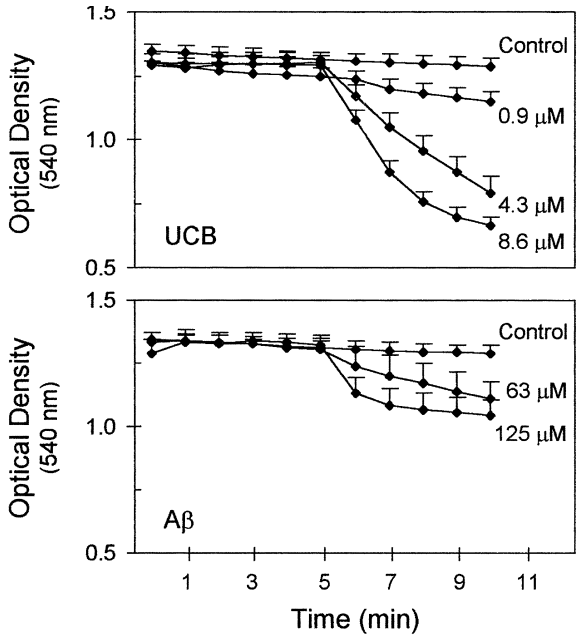

Fig. 3. Dose-response of isolated mitochondria to UCB- and Aβ peptide-induced swelling. Mitochondria were isolated and incubated with either no addition (control), unconjugated bilirubin (UCB; 0.9, 4.3, and 8.6 μM), or amyloid-β (Aβ) peptide (25–35) (63 and 125 μM) in respiration buffer as described in "Materials and Methods." At 5 min, the apoptotic agent was added and the decrease in absorbance at 540 nm was monitored for an additional 5 min-period. The results are means ± standard error of the mean (SEM) of at least three separate experiments.

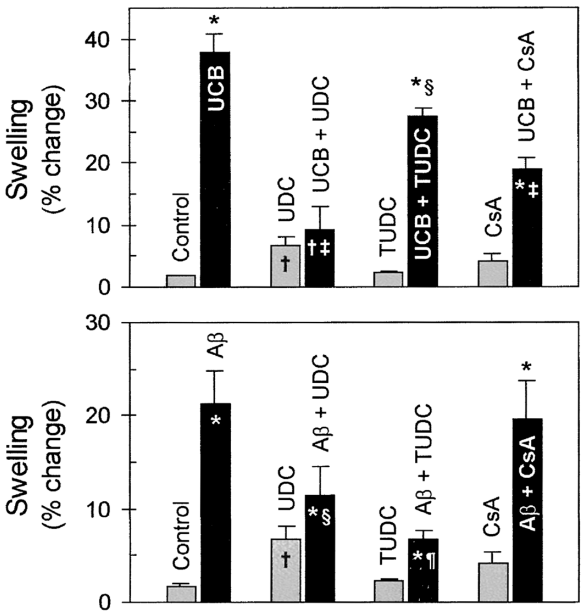

Fig. 4. Percent change in mitochondrial swelling following incubation of isolated mitochondria with UCB and Aβ peptide. Mitochondria were isolated and incubated with either no addition (control), 4.3 μM unconjugated bilirubin (UCB), 125 μM amyloid-β (Aβ) peptide (25–35), or a combination of toxic agent plus 500 μM ursodeoxycholate (UDC), 500 μM taurourso auroursodeoxycholate (TUDC) or 5 μM cyclosprine A (CsA) in respiration buffer as described in "Materials and Methods." At 5 min, the apoptotic agent was added and the percent change in mitochondrial swelling from 5 to 10 min was determined by monitoring the absorbance at 540 nm. In the coincubation experiments, mitochondria were pretreated with UDC, TUDC, or CsA for 5 min. The results are means ± standard error of the mean (SEM) of at least three separate experiments. $^{\dagger}p < 0.05$, $^{*}p < 0.001$ from control; $^{\S}p < 0.05$, $^{\P}p < 0.01$, $^{\ddagger}p < 0.001$ from toxic stimuli alone.

TUDC inhibited Aβ toxicity by 75% ($p < 0.01$), but prevented UCB alterations by only 28% ($p < 0.05$).

The large-volume mitochondrial swelling induced by both UCB and Aβ peptide could be due to either a nonspecific disruption of the mitochondrial membrane or a specific opening of the cyclosporine A-sensitive megapore. Therefore, we next determined if pretreatment of isolated mitochondria with cyclosporine A inhibited UCB- and Aβ-induced mitochondrial swelling (Fig. 4). Similarly to the effect of UDC, cyclosporine A also partially inhibited UCB-mediated swelling by 52% ($p < 0.001$). However, Aβ-induced mitochondrial alterations were only slightly prevented by preincubation with cyclosporine A (9%, not significant; N.S.). Swelling of mitochondria could be explained by the opening of the permeability transition pore during incubation with UCB, but not with Aβ peptide.

UCB and Aβ Peptide Induce Cytochrome c *Release After Membrane Permeabilization in Isolated Mitochondria*

We then determined whether the direct effect of UCB and Aβ peptide on mitochondria to induce the mitochondrial membrane permeability was required for the release of cytochrome *c*. Thus, cytochrome *c* protein levels were determined by Western blot analysis of the mitochondrial pellets and supernatants following incubation with UCB or Aβ peptide. Levels of cytochrome *c* accumulated in supernatants of controls were undetectable (Fig. 5). Likewise, addition of either UDC, TUDC, or cyclosporine A did not significantly influence cytochrome *c* release. In contrast, mitochondria treated with UCB or Aβ peptide showed a marked release of

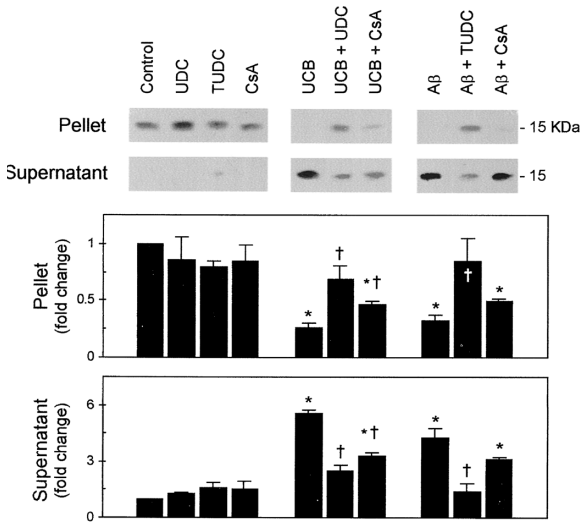

Fig. 5. Cytochrome *c* release following membrane permeabilization in isolated mitochondria incubated with UCB and Aβ peptide. Mitochondria were isolated and incubated with either no addition (control), 4.3 μM unconjugated bilirubin (UCB), 125 μM amyloid-β (Aβ) peptide (25–35), or a combination of toxic agent plus 500 μM ursodeoxycholate (UDC), 500 μM tauroursodeoxycholate (TUDC) or 5 μM (CsA) in respiration buffer as described in "Materials and Methods." Mitochondrial pellets and supernatants were examined for cytochrome *c* levels by Western blot analysis. Following SDS-PAGE and transfer, the nitrocellulose membranes were incubated with the monoclonal antibody to cytochrome *c* and the 15 kDa protein was detected using enhanced chemiluminescent light (ECL) reagent cyclosporine A. Representative Western blots (top) and densitometric means ± standard error of the mean (SEM) relative to controls (bottom) of at least three separate experiments. $^{*}p < 0.01$ from control; $^{\dagger}p < 0.05$ from toxic stimuli alone.

cytochrome *c* ($p < 0.01$), and supernatant levels were proportionally increased ($p < 0.01$). In fact, 4- to 3-fold decreases in protein levels were evident in mitochondrial pellets after incubation with UCB and Aβ, respectively. Addition of either UDC or cyclosporine A prevented UCB-induced efflux of cytochrome *c* by 60 and 27%, respectively ($p < 0.05$). TUDC also prevented cytochrome *c* release associated with Aβ induction of mitochondrial membrane permeability by approximately 80% ($p < 0.05$). Finally, although not significantly, cyclosporine A inhibited mitochondrial efflux of cytochrome *c* induced by Aβ peptide. Thus, cytochrome *c* redistribution appears to be only partially mediated via the mitochondrial permeability transition pore.

Discussion

Evidence suggests that cytotoxicity of UCB and Aβ peptide to neural primary cultures may cause apoptosis. Data presented here support a key role of mitochondria during cell death induced by both toxic stimuli. The involvement of mitochondria is suggested by the demonstration that both UCB and Aβ peptide directly induce membrane permeabilization in isolated mitochondria, that inhibitors of mitochondrial permeability also block UCB and Aβ-induced mitochondrial alterations, and that neural apoptosis is prevented by UDC or its taurine-conjugated derivative.

Bilirubin neurotoxicty presents a major problem in newborn infants, due not only to the high incidence of unconjugated hyperbilirubinemia in this age group, but also to the increased vulnerability of immature neural cells to bilirubin toxicity (44–46). Results from several studies conducted in neural tissues and neuronal cell lines indicate that UCB is toxic to various cellular functions, such as DNA and protein synthesis (14,15,19), modulation of neurotransmitter synthesis, release and uptake (16,20,21), as well as inhibition of protein phosphorylation (17,18,20). In addition, evidence suggests that bilirubin toxicity may be a consequence of mitochondrial impairment. For example, malate-dehydrogenase was inhibited at very low concentrations of bilirubin; whereas, both the mitochondrial and cytosolic forms of aspartate aminotransferase showed moderate competitive bilirubin inhibition (47). Collections of intramitochondrial glycogen were observed in tissue sections of the substancia nigra of hyperbilirubinemic rats (48), which may represent alternate mechanisms of neurons to produce ATP following the dysruption of oxidative phosphorylation by bilirubin. In addition, ligandin, an abundant cytoplasmatic binding protein of bilirubin in liver cells, completely restored respiration and oxidative phosphorylation in isolated rat liver mitochondria following incubation with bilirubin (49). Moreover, incubation of synaptosomal plasma membrane vesicles isolated from rat brain with bilirubin was shown to depress the membrane potential by a mechanism involving alterations in membrane permeability (50). From the present study, incubation of isolated mitochondria with UCB caused mitochondrial alterations associated with membrane permeabilization in a rapid and dose-dependent manner. UCB-induced mitochondrial permeability transition was prevented by either

cyclosporine A, a known inhibitor of the megapore channel, or by UDC, which appears to exhibit an ubiquitous protective effect involving the mitochondrial membrane. The latter also reduced UCB-induced apoptosis in isolated neural cells. These results suggest that the observed mitochondrial permeabilization resulted from perturbation of the cyclosporine A-sensitive large conductance channel, rather than from nonspecific membrane disruption.

Defects in mitochondrial oxidative metabolism have been demonstrated in Alzheimer's disease and after the expression of the amyloid precursor protein in cultured cells, suggesting that mitochondria may be involved in Aβ peptide toxicity (51,52). In particular, incubation of isolated rat mitochondria with Aβ fragment 25–35, but not the reverse sequence peptide, caused a rapid decrease in the activity of the mitochondrial respiratory chain complex IV (53). In contrast, antioxidants inhibited Aβ-induced impairment of energy metabolism preventing the deficit in ATP levels (54). Consequences of electron transport chain dysfunction, such as decreased ATP production and reactive oxygen species production, may lead to further mitochondrial damage, including oxidation of mitochondrial DNA, proteins, and lipids, and opening of the mitochondrial permeability transition pore. As shown in the present study, Aβ peptide directly induces membrane permeabilization in isolated mitochondria through a nonspecific perturbation that can be prevented by either UDC or TUDC. UDC and TUDC also were markedly effective at inhibiting apoptosis in primary cultures of neuronal and glial cells.

The present study also demonstrates that cytochrome *c,* a mitochondrial factor involved in apoptosis, is released from isolated rat mitochondria following disruption of the mitochondrial membrane barrier induced by either UCB or Aβ peptide. Cytochrome *c* redistribution was shown to be a common event in the cell death effector pathway initiated by diverse apoptotic stimuli (55–57), and inhibition of cytochrome *c* activity with blocking antibodies was reported to inhibit apoptosis (58). In fact, a recent study demonstrated that the release of cytochrome green fluorescent protein (c-(GFP)) always precedes exposure of phosphatidylserine and loss of plasma-membrane integrity in apoptotic cells (59). Nevertheless, the precise mechanism of cytochrome *c* efflux from mitochondria into cytosol, and its regulation remain a matter of debate. Several reports have provided evidence that release of the 15 kDa caspase-activating factor occurs independently of any detectable mitocondrial depolarization, implying that opening of the megapore is a downstream event to caspase activation (60). However, data presented here are in agreement with previous work, where mitochondrial permeabilization was shown to coincide with cytochrome *c* relocation, which in turn was required for caspase activation (61,62). Moreover, coincubation of mitochondria with UDC or TUDC resulted in significant inhibition of mitochondrial permeabilization and, therefore, cytochrome *c* release induced by either UCB or Aβ-peptide. The ability of UDC to prevent cytchrome *c* release is not without precedent. In fact, we demonstrated a unique role for UDC in preventing Asp-Glu-Val-Asp (DEVD)-specific caspase activation and poly (ADP-ribose) polymerase cleavage, as well as nuclear fragmentation, which follow efflux of the cytochrome *c* in cells treated with several toxic stimuli (34,35).

In conclusion, our data suggest that UCB and Aβ-peptide directly interfere with membrane permeabilization in isolated mitochondria, thereby, increasing efflux of cytochrome *c,* which may explain the apoptotic effect of these agents in neural cells. Moreover when combined with UCB and Aβ peptide, UDC or its taurine-conjugated derivative play a unique part in regulating neural cell survival through at least one process that appears to involve the mitochondrial membrane. These data provide some evidence that UDC may prove to be an interesting drug for the prevention of apoptosis associated with severe unconjugated hyperbilirubinemia and neurodegenerative disorders, and as an adjunct to standard therapy in the treatment of these diseases.

Acknowledgements

This work was supported by grant PRAXIS/C/SAU/14311/1998 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal, and EASL Research Fellowship from the European Association for the Study of the Liver to CMP Rodrigues.

References

1. Jacobson MD, Weil M, Raff MC. (1997) Programmed cell death in animal development. *Cell* **88:** 347–354.

2. Thompson CB. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456–1462.
3. Evan G, Littlewood T. (1998) A matter of life and cell death. *Science* **281:** 1317–1322.
4. Wyllie AH, Kerr JFR, Currie AR. (1980) Cell death: The significance of apoptosis. *Int. Rev. Cytol.* **68:** 251–306.
5. Columbano A. (1995) Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo. *J. Cell Biochem.* **58:** 181–190.
6. Kroemer G, Zamzami N, Susin SA. (1997) Mitochondrial control of apoptosis. *Immunol Today.* **18:** 44–51.
7. Green DR, Reed JC. (1998) Mitochondria and apoptosis. *Science* **281:** 1309–1312.
8. Wallace DC. (1999) Mitochondrial diseases in man and mouse. *Science* **283:** 1482–1488.
9. Jacobson MD, Burne JF, Raff MC. (1994) Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* **13:** 1899–1910.
10. Schulze-Osthoff K, Walczak H, Droge W, Krammer PH. (1994) Cell nucleus and DNA fragmentation are not required for apoptosis. *J. Cell Biol.* **127:** 15–20.
11. Jacotot E, Costantini P, Laboureau E, Zamzami N, Susin SA, Kroemer G. (1999) Mitochondrial membrane permeabilization during the apoptotic process. *Ann. NY Acad. Sci.* **887:** 18–30.
12. Brodersen R, Stern L. (1990) Deposition of bilirubin acid in the central nervous—a hypothesis for the development of kernicterus. *Acta Paediatr. Scand.* **79:** 12–19.
13. Rubboli G, Ronchi F, Cecchi P, et al. (1997) A neurophysiological study in children and adolescents with Crigler-Najjar syndrome type I. *Neuropediatrics* **28:** 281–286.
14. Majumadar APN. (1974) Bilirubin encephalopathy: effect on RNA polymerase activity and chromatin template activity in the brain of Gunn rat. *Neurobiology* **4:** 425–431.
15. Yamada N, Sawasaki Y, Nakajima H. (1977) Impairment of DNA synthesis in Gunn rat cerebellum. *Brain Res.* **126:** 295–307.
16. Ohno T. (1980) Kernicterus: effect on choline acetyltransferase, glutamic acid decarboxylase and tyrosine hydroxylase activities in the brain of Gunn rat. *Brain Res.* **196:** 282–285.
17. Morphis L, Constantopoulos A, Matsaniotis N. (1982) Bilirubin-induced modulation of cerebral protein phosphorylation in neonate rabbits in vivo. *Science* **218:** 156–158.
18. Sano K, Nakamura H, Matsuo T. (1982) Mode of inhibitory action of bilirubin on protein kinase C. *Pediatr. Res.* **19:** 587–590.
19. Schiff D, Chan G, Poznansky MJ. (1985) Bilirubin toxicity in neuronal cell lines N-115 and NBR-10A. *Pediatr. Res.* **19:** 908–911.
20. Hansen TWR, Bratlid D, Walaas SI. (1988) Bilirubin decreases phosphorylation of synapsin I, a synaptic vesicle-associated neuronal phosphoprotein, in intact synaptosomes from rat cerebral cortex. *Pediatr. Res.* **23:** 219–223.
21. Silva R, Mata LR, Gulbenkian S, Brito MA, Tiribelli C, Brites D. (1999) Inhibition of glutamate uptake by unconjugated bilirubin in cultured cortical rat astrocytes: role of concentration and pH. *Biochem. Biophys. Res. Comm.* **265:** 67–72.
22. Silva R, Rodrigues CMP, Brites D. (In press) Bilirubin-induced apoptosis in glial and nerve cells is aggravated by chenodeoxycholic acid but prevented by ursodeoxycholic acid. *J. Hepatol.*
23. Rodrigues CMP, Solá S, Silva R, Diógenes MJ, Brites D. (2000) Apoptosis induced by deoxycholic acid, unconjugated bilirubin and amyloid β-peptide reflects mitochondrial perturbation which may be inhibited by ursodeoxycholic acid (Abstract). *J. Hepatol.* **32:** 40.
24. Cowger ML, Igo RP, Labbe RF. (1965) The mechanism of bilirubin toxicity studied with purified respiratory enzyme and tissue culture systems. *Biochemistry* **4:** 2763–2770.
25. Mustafa MG, Cowger ML, King TE. (1969) Effects of bilirubin on mitochondrial reactions. *J. Biol. Chem.* **244:** 6403–6414.
26. Noir BA, Boveris A, Garaza Pereira AM, Stoppani AO. (1972) Bilirubin: a multi-site inhibitor of mitochondrial respiration. *FEBS Lett.* **27:** 270–274.
27. Glenner GG. (1988) Alzheimer's disease: its proteins and genes. *Cell* **52:** 307–308.
28. Yankner BA, Duffy LK, Kirschner DA. (1990) Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science* **250:** 279–282.
29. Behl C, Davis J, Cole GM, Schubert D. (1992) Vitamin E protects nerve cells from amyloid beta protein toxicity. *Biochem. Biophys. Res. Commun.* **186:** 944–950.
30. Mattson MP, Cheng *B*, Davis D, Bryant K, Lieberburg I, Rydel RE. (1992) β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.* **12:** 376–389.
31. Su JH, Anderson AJ, Cummings BJ, Cotman CW. (1994) Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport* **5:** 2529–2533.
32. Cotman CW, Anderson AJ. (1995) A potential role for apoptosis in neurodegeneration and Alzheimer's disease. *Mol. Neurobiol.* **10:** 19–45.
33. Rodrigues CMP, Fan G, Ma X, Kren BT, Steer CJ. (1998) A novel role of ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. *J. Clin. Invest.* **101:** 2790–2799.
34. Rodrigues CMP, Ma X, Linehan-Stieers C, Fan G, Kren BT, Steer CJ. (1999) Ursodeoxycholic acid

prevents cytochrome *c* release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. *Cell Death Differ.* **6:** 842–854.
35. Rodrigues CMP, Keene CD, Linehan-Stieers C, Ma X, Low W, Steer CJ. (2000) Tauroursodeoxycholic acid prevents apoptosis induced by the neurotoxin 3-nitropropionic acid in rat neuronal cells: evidence for a mitochondrial-dependent pathway that does not involve the permeability transition (Abstract). *J. Hepatol.* **32:** 86.
36. Brewer GJ, Torricelli JR, Evege EK, Price PJ. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35:** 567–576.
37. Blondeau JP, Beslin A, Chantoux F, Francon J. (1993) Triiodothyronine is a high-affinity inhibitor of amino acid transport system L1 in cultured astrocytes. *J. Neurochem.* **60:** 1407–1413.
38. McDonagh AF, Assisi F. (1972) The ready isomerization of bilirubin IX- in aqueous solution. *Biochem. J.* **129:** 797–800.
39. Oberhammer FA, Pavelka M, Sharma S, et al. (1992) Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β 1. *Proc. Natl. Acad. Sci. USA* **89:** 5408–5412.
40. Walajtys-Rhode E, Zapatero J, Moehren G, Hoek JB. (1992) The role of the matrix calcium level in the enhancement of mitochondrial pyruvate carboxylation by glucagon pretreatment. *J. Biol. Chem.* **267:** 370–379.
41. Botla R, Spivey JR, Aguilar H, Bronk SF, Gores GJ. (1995) Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection. *J. Pharmacol. Exp. Ther.* **272:** 930–938.
42. Sokol RJ, Devereaux M, Mierau GW, Hambidge KM, Shikes RH. (1990) Oxidant injury to hepatic mitochondrial lipids in rats with dietary copper overload. Modification by vitamin E deficiency. *Gastroenterology* **99:** 1061–1071.
43. Rodrigues CM, Fan G, Wong PY, Kren BT, Steer CJ. (1998) Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. *Mol. Med.* **4:** 165–178.
44. Amit Y, Brenner T. (1993) Age-dependent sensitivity of cultured rat glial cells to bilirubin toxicity. *Exp. Neurol.* **121:** 248–255.
45. Hansen TW, Allen JW. (1997) Oxidation of bilirubin by brain mitochondrial membranes-dependence on *Cell* type and postnatal age. *Biochem. Mol. Med.* **60:** 155–160.
46. Rhine WD, Schmitter SP, Yu AC, Eng LF, Stevenson DK. (1999) Bilirubin toxicity and differentiation of cultured astrocytes. *J. Perinatol.* **19:** 206–211.
47. McLoughlin DJ, Howell ML. (1987) Bilirubin inhibition of enzymes involved in the mitochondrial malate-aspartate shuttle. *Biochim. Biophys. Acta* **893:** 7–12.
48. Batty HK, Millhouse OE. (1976) Ultrastructure of the Gunn rat substantia nigra. II. Mitochondrial changes. *Acta Neuropathol.* **34:** 7–19.
49. Kamisaka K, Gatmaitan Z, Moore CL, Arias IM. (1975) Ligandin reverses bilirubin inhibition of liver mitochondrial respiration in vitro. *Pediatr. Res.* **9:** 903–905.
50. Mayor F. Diez-Guerra J, Valdivieso F, Mayor F. (1986) Effect of bilirubin on the membrane potential of rat brain synaptosomes. *J. Neurochem.* **47:** 363–369.
51. Beal MF. (1998) Mitochondrial dysfunction in neurodegenerative diseases. *Biochim. Biophys. Acta* **1366:** 211–223.
52. Christen Y. (2000) Oxidative stress and Alzheimer disease. *Am. J. Clin. Nutr.* **71:** 621S–629S.
53. Canevari L, Clark JB, Bates TE. (1999) β-Amyloid fragment 25-35 selectively decreases complex IV activity in isolated mitochondria. *FEBS Lett.* **457:** 131–134.
54. Prasad KN, Cole WC, Hovland AR, et al. (1999) Multiple antioxidants in the prevention and treatment of neurodegenerative disease: analysis of biologic rationale. *Curr. Opin. Neurol.* **12:** 761–770.
55. Kim CN, Wang X, Huang Y, et al. (1997) Overexpression of Bcl-X_L inhibits Ara-C-induced mitochondrial loss of cytochrome *c* and other perturbations that activate the molecular cascade of apoptosis. *Cancer Res.* **57:** 3115–3120.
56. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275:** 1132–1136.
57. Yang J, Liu X, Bhalla K, et al. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* **275:** 1129–1132.
58. Neame SJ, Rubin LL, Philpott KL. (1998) Blocking cytochrome *c* activity within intact neurons inhibits apoptosis. *J. Cell Biol.* **142:** 1583–1593.
59. Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. (2000) The coordinate release of cytochrome *c* during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* **2:** 156–162.
60. Bossy-Wetzel E, Newmeyer DD, Green DR. (1998) Mitochondrial cytochrome *c* release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J.* **17:** 37–49.
61. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* **86:** 147–157.
62. Li P, Nijhawan D, Budihardjo I, et al. (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91:** 479–489.