Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells

Ana S. Falcão, Adelaide Fernandes, Maria A. Brito, Rui F.M. Silva, and Dora Brites*

Centro de Patogênese Molecular (UBMBE), Faculdade de Farmácia, University of Lisbon, Av. Forças Armadas, 1600-083 Lisboa, Portugal

Received 25 January 2005; revised 25 February 2005; accepted 3 March 2005
Available online 12 April 2005

Unconjugated bilirubin (UCB) encephalopathy is a predominantly early life condition resulting from the impairment of several cellular functions in the brain of severely jaundiced infants. However, only few data exist on the age-dependent effects of UCB and their association with increased vulnerability of premature newborns, particularly in a sepsis condition. We investigated cell death, glutamate efflux, and inflammatory cytokine dynamics after exposure of astrocytes at different stages of differentiation to clinically relevant concentrations of UCB and/or lipopolysaccharide (LPS). Younger astrocytes were more prone to UCB-induced cell death, glutamate efflux, and inflammatory response than older ones. Furthermore, in immature cells, LPS exacerbated UCB effects, such as cell death by necrosis. These findings provide a basis for the increased susceptibility of premature newborns to UCB deleterious effects, namely when associated with sepsis, and underline how crucial the course of cell maturation can be to UCB encephalopathy during moderate to severe neonatal jaundice.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Astrocytes; Development; Cell death; Glutamate release; Lipopolysaccharide (LPS); Cytokines; Unconjugated bilirubin; Cell vulnerability

Introduction

Hyperbilirubinemia is a common condition in the neonatal period as a result of decreased erythrocyte survival and defective hepatic clearance of unconjugated bilirubin (UCB) (Dennery et al., 2001). Premature infants, when compared to full-term newborns, have higher rates of UCB production as a result of an even shorter life span of their red blood cells (Stevenson et al., 2001). In addition, prematures present a markedly decrease of both hepatic UDP-glucuronosyltransferase activity (Kawade and Onishi, 1981) and uptake and secretion of bilirubin (Obrinsky et al., 1952; Vest and Rossier, 1963). The significance of this neonatal jaundice can range from an acute bilirubin encephalopathy, seen in the first weeks and transiently affecting the central nervous system (CNS) function, to a more severe condition called kernicterus, with chronic and permanent neurologic sequelae as a result of UCB neurotoxicity. In the later case, surviving infants may develop severe forms of auditory and mental dysfunction or other handicaps (American Academy of Pediatrics, 2004).

Recently, the pathological conditions associated with hyperbilirubinemia were brought to daylight again as kernicterus reemerged as a risk factor for infants in countries where this complication had essentially disappeared (Ebbesen, 2000; Hansen, 2000). Early hospital discharge of term infants and increased survival of prematurely born infants might be implicated in this reemergence (Gourley, 1997). The evidence of minor neurologic dysfunctions throughout the first year of life in children that have presented a moderate neonatal hyperbilirubinemia (Soorani-Lunsing et al., 2001) indicated that the serum bilirubin level is not the only hazardous factor to consider when clinicians evaluate a jaundiced newborn. Therefore, there is now a growing concern to clarify the underlying mechanisms of UCB neurotoxicity and to elucidate the role of other risk factors in order to identify the infants who are more vulnerable to neurological damage (American Academy of Pediatrics, 2004).

The numerous studies conducted in recent years to disclose the molecular mechanisms of UCB neurotoxicity have shown that this molecule interacts with cell membranes (Brito et al., 2000, 2001), accumulates within the cells, and disrupts several vital functions (Brito et al., 2004; Rodrigues et al., 2000; Silva et al., 2001), having more than one particular mechanism of toxicity (Ostrow et al., 2004).

We have previously shown that neurons exposed to UCB reveal higher susceptibility to cell death by necrosis and apoptosis while astrocytes are increasingly impaired in cell function (Silva et al., 2002). Recently, we demonstrated, for the first time, that a short exposure to UCB leads to an increase of extracellular glutamate and highly enhances the release of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, while it inhibits the production of IL-6. Moreover, co-incubation with lipopolysaccharide (LPS), used to mimic infection, further enhanced the secretion of proinflammatory cytokines, without changing either the release of glutamate or IL-6.
secretion (Fernandes et al., 2004). Thus, cytokines seem to play a key role in UCB-induced cell death, in which glutamate might act cooperatively. Moreover, endotoxin can aggravate the immunostimulant effects of UCB. Therefore, it might be important to investigate these effects in immature neural cells as an approach to what happens in jaundiced preterm newborns, particularly when there is sepsis. In fact, premature newborns have an increasing susceptibility to UCB toxic effects during moderate to severe neonatal jaundice (Gourley, 1997; Dennerly et al., 2001).

Besides the low birth-weight and hypothybunemia of premature infants, they may as well be affected by insults like acidosis, hypothermia, and sepsis, which have been considered as significant aggravating factors (Lucey, 1972), probably by altering the blood–brain barrier or enhancing cellular uptake of UCB (Ritter et al., 1982). Additionally, prematurity is very often associated with anoxic–ischemic insults, which can also cause severe brain damage (Alhab-Barmada and Moossy, 1984). Thus, when studying the mechanisms of UCB toxicity, immaturity of cells is an important issue to consider. Indeed, we have already demonstrated that in UCB-exposed astrocytes and neurons, cell death is higher in younger than in older cells (Rodrigues et al., 2002). Likewise, younger erythrocytes are significantly more susceptible to UCB-induced morphological alterations than older ones (Brites et al., 1997). Hence, we can hypothesize that the higher vulnerability of premature infants to UCB neurotoxicity may be due to proneness of immature neural cells to neurotoxic injury.

The aims of this study were to (1) assess age-related differences in cell death, glutamate efflux, and inflammatory cytokine dynamics, after exposure of astrocytes to clinically relevant concentrations of UCB, in order to explain the potentially greater toxicity of the pigment to immature nervous cells; (2) further elucidate the effect of sepsis in UCB neurotoxicity and its putative role in the increased susceptibility of premature newborns. Our results show that younger astrocytes are more prone to UCB-induced cell death, glutamate efflux, and inflammatory response than older ones. We further demonstrate that in immature cells, LPS exacerbates some of these deleterious effects of UCB, namely cell death by necrosis.

**Experimental procedures**

**Animals and cell culture**

Wistar rats were maintained on a 12-h light/dark cycle under conditions of constant temperature and humidity. Animals were supplied with standard laboratory chow and water ad libitum. The Institutional Animal Ethics Committee approved the study protocol and all procedures complied with international standards of humane care in animal experimentation.

Astrocytes were isolated from 2-day-old rats as previously described (Blondeau et al., 1993), with minor modifications (Silva et al., 1999). Briefly, rats were decapitated and the brains collected in Dulbecco’s modified Eagle’s medium (DMEM; Biochrom AG, Germany) containing 11 mM sodium bicarbonate, 71 mM glucose, and 1% antibiotic antimycotic solution (Sigma, St. Louis, MO). The cortical fraction was homogenized by mechanical fragmentation, and a cell suspension, collected after centrifugation (10 min at 700 × g), was resuspended in culture medium supplemented with 10% fetal calf serum (FCS; Biochrom AG). Finally, 2.0 × 10^5 cells/cm^2 were plated on 12-well tissue culture plates (Corning Costar Corp., USA) and maintained at 37°C in a humidified atmosphere of 5% CO₂. Culture medium was replaced at day 7 and then every 3 days. Cells were morphologically characterized by phase contrast microscopy and days in vitro (DIV) were counted from the first day of cell seeding. All cultures were analyzed at 5, 10, and 20 DIV. This model of aging in vitro is based on the fact that the timing of development of glial cells is similar in dissociated cell cultures and in vivo (Abney et al., 1981) and that full term newborns correspond best to 12- to 13-day-old rats (Romijn et al., 1991). In this way, in our cultures, 10 DIV astrocytes will correspond to a term infant, and therefore, less differentiated cells to a premature stage.

**Astrocyte treatment**

UCB was purified according to McDonagh and Assisi’s (1972) method and all UCB solutions prepared from a 10-mM stock solution in 0.1 N NaOH, and used briefly after preparation, in diminished light conditions. HCl 0.1 N was used to restore the pH value to 7.4. LPS was dissolved in PBS at 1 mg/mL.

Cultured cells were incubated for 4 h with 50 μM UCB in the presence of 100 μM human serum albumin (HSA; Sigma) (molar ratio of 0.5), or with 1 ng/mL Escherichia coli O111:B4 lipopolysaccharide (LPS; Calbiochem, La Jolla, CA, USA), at 37°C. For co-incubation studies, astrocytes were exposed to UCB and LPS. Controls were performed in the absence of UCB and LPS. At the end of the incubation periods, cell-free medium was collected for LDH, glutamate, and cytokine determinations, while attached cells were fixed for 30 min with freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for both apoptosis and immunocytochemical studies.

**Cell death**

The assay used to estimate cell death was based on the release of lactate dehydrogenase (LDH) by viable cells. LDH was determined in the incubation medium using the Cytotoxicity Detection kit, LDH (Roche Molecular Biochemicals, Mannheim, Germany). All readings were corrected for the possible interference of UCB absorption and the results were expressed as percent of total LDH release, obtained by treating non-incubated cells with 2% Triton X-100 in DMEM for 5 min. Apoptosis was evaluated by assessment of nuclear morphology. In brief, astrocytes were incubated with Hoechst dye 33258 (Sigma) at 5 μg/mL in PBS, for 2 min at room temperature, washed with PBS, and mounted using PBS/glycerol (3:1, v/v). Fluorescence was visualized using an Axioskop® microscope (Zeiss, Germany). Apoptotic nuclei were identified by condensed chromatin, as well as nuclear fragmentation, and were counted in at least five random microscopic fields (×400) per sample. The mean values were expressed as the percentage of apoptotic nuclei.

**Immunocytochemistry**

Microglial contamination in astrocytes cultured for 5, 10, and 20 DIV was assessed by immunocytochemical staining using primary antibodies raised against OX-42 (mouse, 1:200; Serotec, Raleigh, NC, USA) and GFAP (rabbit, 1:100; Sigma), for microglia and astrocyte labeling, respectively, followed by a species-specific fluorescent secondary antibody labeled with FITC for OX-42 (Vector, Burlingame, CA, USA) or with TRITC for GFAP (Sigma).
Cytokine determinations

Culture supernatants free from cellular debris were assessed in duplicate for TNF-α, IL-1β, and IL-6 with specific Quantikine ELISA kits from R&D Systems, Inc (Minneapolis, MN, USA), using a microplate reader PR 2100 (Bio-Rad Laboratories, Hemel Hempstead, UK), according to the manufacturer’s instructions. Results were expressed in pg/mL and normalized to 10^5 cells for each culture.

Measurement of glutamate

Release of glutamate to the culture medium was determined by an adaptation of the L-Glutamic acid Kit (Roche) using a 10-fold reduction in kit reagents. Samples were pipetted to a 96-well plate and read in the microplate reader (Bio-Rad Laboratories) at 490 nm using a reference filter of 620 nm. A new calibration curve was used in each assay and the results normalized to 10^5 cells.

Statistical analysis

Results of, at least, three different experiments, performed in duplicate, were expressed as mean ± SEM. Differences between groups were compared using the two-tailed t test on the basis of equal or unequal variance, as appropriate. We considered P < 0.05 to be statistically significant.

Results

Immature astrocytes are more prone to cell death and more susceptible to UCB and LPS toxicity

We examined whether age-dependent pattern in cultured astrocytes determines cell responsiveness to UCB and LPS injury, either alone or in association. Firstly, we evaluated cell integrity as a function of age by measuring LDH release after 4-h exposure in either alone or in association. Firstly, we evaluated cell integrity as astrocytes determines cell responsiveness to UCB and LPS injury, mainly increased in cultures of younger astrocytes exposed to UCB. To test this, we measured glutamate in cultured media of 5, 10, and 20 DIV astrocytes after 4-h incubation in control conditions, as well as with 50 µM UCB in the absence or in the presence of 1 ng/mL LPS. In baseline controls, the release of glutamate decreased 2.8-fold from 5 to 10 DIV (P < 0.01) and 2.4-fold from 10 to 20 DIV (P < 0.05) (Fig. 2, Table 1). Thus, these results evidence once again an increased response of the youngest cells that was also noticed when incubated with UCB, where the highest levels in glutamate release (21.7 nmol/10^5 cells, P < 0.01) were achieved in the less differentiated cells. In marked contrast, LPS treatment was unable to enhance glutamate release or to modify the UCB effect, confirming the results recently obtained by our group for 10 DIV cells (Fernandes et al., 2004).

TNF-α and IL-1β secretion are highest in less differentiated astrocytes and increase after exposure to UCB and LPS

To investigate cell-age-dependent pattern of inflammatory response, we first determined the purity of astrocyte cultures, since microglia are the major brain cells producing cytokines. The purity level of our astrocyte cultures varied from 99.0% at 5 DIV to 96.5% at 20 DIV (Fig. 3). These results exclude the interference of contaminant microglial cells that could be responsible for either the secretion of cytokines or the glutamate release (Hansich, 2002; Nakamura et al., 2003).

Basal levels of TNF-α decreased with age in culture (Fig. 4, Table 2). In addition, although production of this proinflammatory cytokine following astrocyte exposure to UCB or LPS clearly increased at all ages, the total amount was much higher in 5 DIV.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>5 DIV vs. 10 DIV</th>
<th>5 DIV vs. 20 DIV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P value</strong></td>
<td><strong>P value</strong></td>
<td></td>
</tr>
<tr>
<td>LDH release</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UCB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UCB + LPS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UCB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS + UCB</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>n.s.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UCB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS + UCB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Cells were incubated as described in Experimental procedures. n.s., not significant.

than in 10 or 20 DIV, either for UCB (2.7- and 4.3-fold, respectively, \( P < 0.01 \)) or LPS (2.8- and 6.3-fold, respectively, \( P < 0.01 \)). Co-treatment with UCB and LPS further induced the secretion of TNF-\( \alpha \), but only significantly at 10 DIV, when compared to UCB alone (\( P < 0.05 \)). Similar to TNF-\( \alpha \), but less evident, the release of IL-1\( \beta \) was also enhanced at the early stage in vitro (Fig. 5) and decreased for 10 and 20 DIV, but less markedly than previously reported for TNF-\( \alpha \). An increase of the concentration of IL-1\( \beta \) in cultured supernatants after exposure of astrocytes to UCB or LPS was observed irrespective of age, but clear differences were only noticed between the youngest and the oldest cells (\( P < 0.01 \)). In addition, co-treatment with UCB and LPS further increased the secretion of IL-1\( \beta \) at 10 DIV as compared to UCB alone (\( P < 0.05 \)).

**Immunosuppressive effects of UCB on IL-6 secretion remain significant along astrocyte differentiation**

We have recently shown that exposure of astrocytes to UCB leads to the inhibition of IL-6 secretion in 10 DIV cell cultures (Fernandes et al., 2004). Therefore, we analyzed whether this effect was reduced or abolished in immature cells that revealed to be particularly able to secrete proinflammatory cytokines in accordance to the previous results. IL-6 baseline in control conditions significantly decreased with age in culture (Fig. 6, Table 2) and, as we have hypothesized, inhibition of the IL-6 release by UCB was less marked in cells at 5 DIV (1.4-fold) than at 10 and 20 DIV (1.6- and 1.5-fold, respectively). Upon stimulation with 1 ng/mL LPS, 10 DIV cells demonstrated the highest response (1.3-fold, \( P < 0.05 \)), and consequently, lower levels were obtained either in younger (1.1-fold) or in older cells (1.2-fold). Interestingly, these immunostimulant effects of LPS did not prevail in the presence of UCB.

**Discussion**

Our previous studies have shown that treatment of 10 DIV astrocytes with UCB leads to accumulation of extracellular glutamate and to the release of proinflammatory cytokines TNF-\( \alpha \) and IL-1\( \beta \), as well as suppression of the secretion of IL-6 (Fernandes et al., 2004). Additionally, this work provided some evidence that endotoxin enhances the UCB-induced cell death and release of proinflammatory cytokines, supporting the association of infection with an increased risk of UCB encephalopathy. Another risk factor that is important to consider in UCB-induced brain damage is the prematurity of neonates, a condition that increases the vulnerability to neurotoxins. Therefore, with the present work, we extended our previous studies to provide supportive evidence that this higher propensity of premature newborns to UCB brain damage is, in part, related to a proneness of immature cells to UCB and LPS, alone or in combination.

The results presented here reinforce the concept that UCB-induced cell death in neurons and astrocytes is modulated by age in culture, with immature cells presenting the greatest susceptibility. This is in accordance with the age-dependent sensitivity to UCB toxicity first described by Amit and Brenner (1993) and also with our previous findings in cells at different stages of differentiation (Rodrigues et al., 2002).

In addition, it is also known that caspase-3, a protease involved in the apoptotic program, is strongly activated in less differentiated cells (Lesuisse and Martin, 2002; Xu et al., 2004), suggesting that immaturity is, by itself, an activation factor of apoptosis that might explain the greater values of cell death found in 5 DIV. The same is true for in vivo studies, which demonstrate that caspase-3 is highly expressed in the developing nervous system (de Bilbao et al., 1999; Roth and D’Sa, 2001; Xu et al., 2004; Yakovlev et al., 2001).

In immature astrocytes, there was also an increased level of extracellular glutamate. It is known that elevated release of glutamate and the consequent excessive activation of its receptors trigger excitotoxicity (Olney, 1971), astrogliosis (Martinez-Conteras et al., 2002), and neuronal cell death, particularly when cells are coincidentally subjected to adverse conditions such as hypoxia, ischemia, increased levels of oxidative stress, exposure to toxins, or other pathogenic agents (Mattson, 2003). Some authors already noticed that glutamate uptake also depends on age and that older cells are not only the most efficient (Gottfried et al., 2002) but also present a higher expression of glutamate transporters (Brunet et al., 2004). Additionally, since UCB inhibits glutamate uptake in astrocytes (Silva et al., 1999, 2002), UCB-treated immature cells will be increasingly exposed to high levels of extracellular glutamate and consequently to a greater excitotoxic damage. This
issue is very important if it occurs in the immature brain, since this UCB-induced excitotoxicity might affect neuronal survival of the newborn and increase the risk of brain damage and long-term adverse neurodevelopmental effects.

The relative increase in cytokine production by astrocyte exposure to UCB was almost the same along the maturation of cells, but quantitatively, there was a decrease on the cytokine content of the culture medium as cells grow old. These high levels of cytokines observed in the first days may be related with the fact that cytokines are involved in CNS development. In fact, several studies have tried to understand how cytokines affect the proliferation, survival, and differentiation of neural cells during development (Muñoz-Fernández and Fresno, 1998; Zhao and Schwartz, 1998). IL-1β has been reported to modulate neuron and glial cell survival and growth during development (Brenneman et al., 1992; Giulian et al., 1988a). Interestingly, this cytokine appears in the prenatal brain at the same time as astrocytes and acts directly as a growth factor for these cells in the developing brain (Giulian et al., 1988b). In fact, the maintenance of IL-1β secretion from 5 to 10 DIV, observed in baseline control levels, may be related to the fact that these cells are still in a proliferating stage, reaching a more differentiated one at 20 DIV, where the pattern of cytokine production then decreases.

As IL-1β, TNF-α has also been described to be involved in the development of the CNS, being highly expressed in the embryonic stage (Mehler and Kessler, 1997; Yamasu et al., 1989). This cytokine induces proliferation on primary astrocytes (Barna et al., 1990; Selmaj et al., 1990) and can stimulate the synthesis of neurotrophic factors, such as the nerve growth factor by these cells (Gadient et al., 1990). Our results point out that this cytokine is highly secreted in the immature stage and it decreases till 10 DIV,
Table 2
Statistical differences (P value) in astrocyte inflammatory response between 5 and 10 or between 5 and 20 DIV in cells treated with either no addition or with UCB and LPS, alone or in association

<table>
<thead>
<tr>
<th></th>
<th>5 DIV vs. 10 DIV</th>
<th>5 DIV vs. 20 DIV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>P value</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UCB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS + UCB</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>n.s.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>n.s.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UCB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS + UCB</td>
<td>n.s.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>n.s.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UCB</td>
<td>n.s.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPS + UCB</td>
<td>n.s.</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Cells were incubated as described in Experimental procedures. n.s., not significant.

where it stabilizes, suggesting an earlier action or a shorter effect on astrocyte proliferation, as compared to IL-1β.

In the early development, both IL-1β and TNF-α can induce IL-6 synthesis in astrocytes, which in turn has been proposed to function as a developmental neurotrophic factor (Gadient and Otten, 1994), being detected in rat cortex at embryonic day 18, or possibly earlier (Pousset, 1994). Thus, we can postulate that, because of this greater induction in the developing stage, the UCB-induced decrease in IL-6 production at 5 DIV had a lower variation when comparing with 10 and 20 DIV.

Although it was clearly demonstrated that cytokine secretion is developmentally regulated in astrocytes, neither the exact mechanisms underlying the immunostimulant and immunosuppressive effects of UCB nor the neuroprotective or neurotoxic properties of the immune response have been clarified.

An enhanced cytokine production in the immature stage was also demonstrated in in vivo studies, where a greater cytokine synthesis was found in term and preterm neonates, as compared to adults (Schultz et al., 2002), refuting the initial idea that premature infants are unable to produce an inflammatory response because of their immaturity (Chang et al., 1994; Pillay et al., 1994; Schibler et al., 1992).

What are the mechanisms behind this enhanced production of cytokines in immature cells? It is well studied that during CNS development, there is a coordinated expression of multiple genes that are controlled by specific transcription factors (Bergeron et al., 1998). Transcription factors such as the nuclear factor kappa B (NF-κB), Fos/Jun, and CREB are developmentally regulated in rat brain, being the highest activity found during the late prenatal and early postnatal days (Alcantara and Greenough, 1993; Bakalkin et al., 1993; Cauley and Verma, 1994; Pennypacker et al., 1993, 1995). Among these factors, NF-κB may provide the link between early signaling events and gene expression. The target genes for NF-κB in brain encode for proteins with immune and inflammatory activities, namely cytokines such as TNF-α, IL-1β, and IL-6 (O’Neill and Kaltschmidt, 1997). Thus, the higher production of these cytokines in immature cells may be due to a developmentally regulated pattern of NF-κB activation (Bakalkin et al., 1993; Cauley and Verma, 1994).

In our previous work (Fernandes et al., 2004), we used LPS to evaluate whether sepsis aggravates the responsiveness of 10 DIV astrocytes to UCB. We concluded that endotoxin had no effect on glutamate release, but enhanced the UCB-induced necrosis of astrocytes, as well as the release of the proinflammatory cytokines TNF-α and IL-1β. Corroborating these results, it has been shown that maternal endotoxin administration causes a dose-dependent cytokine release in the rat fetal brain (Cai et al., 2000) and also that LPS-induced inflammatory response is characterized by great increases in TNF-α and IL-1β concentrations in the neonatal rat brain, 6 h after LPS injection (Cai et al., 2003). Altogether, these released cytokines can mediate the blood–brain barrier breakdown (Didier et al., 2003), inducing brain injury and rendering higher concentrations of UCB in the CNS. The effects of these cytokines may be even more hazardous when considering the increased vulnerability of the premature infant, characterized by an immature blood–brain barrier, incomplete myelination, and suboptimal levels of endogenous protectors (Dammann and Leviton, 1999). However, in our in vitro aging model, and regarding immature cells, LPS only significantly increased apoptosis and TNF-α secretion and, faced with UCB, it only had the ability to significantly increase cell death by

Fig. 5. IL-1β secretion by astrocytes at 5, 10, and 20 DIV. Cells were incubated with 1 ng/mL LPS, 50 μM UCB, UCB plus LPS, or no addition (Control), in the presence of 100 μM of HSA. *P < 0.05, **P < 0.01 vs. respective control. 1P < 0.05 vs. UCB alone.

Fig. 6. IL-6 secretion by astrocytes at 5, 10, and 20 DIV. Cells were incubated with 1 ng/mL LPS, 50 μM UCB, UCB plus UCB, or no addition (Control), in the presence of 100 μM of HSA. *P < 0.05, **P < 0.01 vs. respective control.
necrosis, probably because of the fact that these cells were already under a very hazardous environment. Nevertheless, these results point out that hyperbilirubinemia should be more cautiously monitored in premature newborns, particularly when infection by bacteria is anticipated.

In conclusion, our results point out that immature astrocytes are more prone to UCB-induced cell death, glutamate efflux, and inflammatory response than more differentiated ones. Additionally, in the early cultures, LPS exacerbates some of the deleterious effects of UCB, namely cell death by necrosis. At this point, it will be interesting to study the mechanisms that regulate this higher vulnerability of immature cells in our model, as well as those underlying UCB and LPS toxicity, and how they are aggravated at this early stage of differentiation. The elucidation of these mechanisms will provide a basis for the increased susceptibility of premature newborns and a further insight into the pathogenesis of UCB-induced neurotoxicity that occurs in immature brain, offering a starting point for the development of new therapeutic interventions.

Acknowledgments

This work was supported in part by grant FCT-POCTI/39906/FCT/2001 from Fundação para a Ciência e a Tecnologia (to D.B.), Lisbon, Portugal.

References


