Bilirubin Exerts Additional Toxic Effects in Hypoxic Cultured Neurons from the Developing Rat Brain by the Recruitment of Glutamate Neurotoxicity

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ABSTRACT

Both hypoxia and bilirubin are common risk factors in newborns, which may act synergistically to produce anatomical and functional disturbances of the CNS. Using primary cultures of neurons from the fetal rat brain, it was recently reported that neuronal apoptosis accounts for the deleterious consequences of these two insults. To investigate the influence of hypoxia, bilirubin, or their combination on the outcome of neuronal cells of the immature brain, and delineate cellular mechanisms involved, 6-d-old cultured neurons were submitted to either hypoxia (6 h), unconjugated bilirubin (0.5 μ M), or to combined conditions. Within 96 h, cell viability was reduced by 22.7% and 24.5% by hypoxia and bilirubin, respectively, whereas combined treatments decreased vital score by 34%. Nuclear morphology revealed 13.4% of apoptotic cells after hypoxia, 16.2% after bilirubin, and 22.6% after both treatments. Bilirubin action was specifically blocked by the glutamate receptor antagonist MK-801, which was without effect on the consequences of hypoxia. Temporal changes in [3 H]leucine incorporation rates as well as beneficial effects of cycloheximide reflected a programmed phenomenon dependent upon synthesis of selective proteins. The presence of bilirubin reduced hypoxia-induced alterations of cell energy metabolism, as reflected by 2-D-[3 H]deoxyglucose incor-

poration, raising the question of free radical scavenging. Measurements of intracellular radical generation, however, failed to confirm the antioxidant role of bilirubin. Taken together, our data suggest that low levels of bilirubin may enhance hypoxia effects in immature neurons by facilitating glutamate-mediated apoptosis through the activation of *N*-methyl-D-aspartate receptors. (*Pediatr Res* 49: 507–513, 2001)

Abbreviations

CHX, cycloheximide
DAPI, 4,6-diamidino-2-phenylindole
2-DG, 2-D-deoxyglucose
DHR, dihydrorhodamine 123
MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo, [a,d]-cycloheptene-5,10-imine maleate
MTT, 3-[4,5-dimethylthiazol, 2-yl]-2,5-diphenyltetrazolium bromide
NBQX, 1,2,3,4-tetrahydroxy-6-nitro-2,3-dioxo-benzo(f)quinoxaline, 7-sulfonamide isodium
NMDA, *N*-methyl-D-aspartate
TCA, trichloroacetic acid

Cerebral hypoxia remains a significant risk for neonatal morbidity and mortality, as well as long-term neurologic sequelae such as cerebral palsy, mental retardation, or seizure disorders (1, 2). Although lack of oxygen has been associated with neuronal degeneration (3, 4), it was not clear whether hypoxia itself, without the influence of combined ischemia, kills brain cells. In contrast to studies in intact animals, which are often difficult to interpret due to a number of confounding variables, cell culture seems a useful tool for elucidating cellular and molecular mechanisms involved in clinical disor-

ders such as cerebral hypoxia (5, 6). Indeed, neuronal cells cultured in chemically defined medium have proven to mimic morphologic, electrophysiological, and neurochemical development and differentiation occurring *in vivo* (7, 8), and such a preparation provides the advantage of investigating cellular responses inherent to a single variable. By using cultured neurons from the developing rat forebrain as a model, we have previously demonstrated that transient oxygen deprivation may trigger neuronal death through an apoptotic process that requires specific and time-dependent changes in protein synthesis (9, 10), a conclusion supported by other studies (6, 11).

Bilirubin constitutes another potential cause of brain injury (12, 13). Hyperbilirubinemia is still a common concern in neonatology, especially in the care of low birth weight prema-

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ture infants. Interestingly, it has been reported that bilirubin can be present in the brains of infants who did not display significant elevations in serum bilirubin concentrations (14). In this respect, a recent study from our laboratory has shown in the same culture model that low concentration of unconjugated bilirubin (*i.e.* 0.5 μM) induces neuronal apoptosis involving caspase activation and that would be mediated by the NMDA glutamate receptor subtypes (15).

A number of neuropathological links between cerebral hypoxia and bilirubin have been described. Since the pioneering animal study by Lucey *et al.* (16) showing that antecedent hypoxic damage of the brain was prerequisite to the subsequent occurrence of kernicterus in the presence of hyperbilirubinemia, numerous clinical reports have emphasized the role of perinatal asphyxia, together with prematurity, acidosis, respiratory distress syndrome, and infection, as well as the use of certain drugs, as predisposing risk factors for bilirubin encephalopathy in newborn infants with low levels of serum bilirubin (17–19). Neurologic damage associated with perinatal asphyxia shows brain regional distribution and clinical manifestations somewhat similar to those observed in bilirubin encephalopathy (20–22). Furthermore, severe postnatal hypoxia has been reported to significantly increase bilirubin brain levels in newborn rats (23), with preferential bilirubin accumulation in cerebral areas corresponding to those more severely injured as a result of perinatal asphyxia (24). Also, the incidence of hypoxic encephalopathy in neonates was more elevated in kernicteric than in normal infants (25), suggesting that bilirubin may favor hypoxic brain damage.

Various signals may trigger neuronal apoptosis that results in similar final manifestations of cell death, and the available evidence indicates that several biochemical pathways may be involved in the apoptotic cascade (26). This suggests that distinct apoptosis-inducing agents may act complementarily and thus exert additive neurotoxic effects. The present study was therefore designed in an attempt to compare the influence of hypoxia and bilirubin on the outcome of neuronal cells *in vitro*, and analyze the cellular mechanisms that may contribute to the deleterious effects of low levels of bilirubin in conjunction with transient hypoxia.

METHODS

Neuronal cell culture. All animal experimentation was performed with the standards of animal care and housing, according to the *NIH Guide for the Care and Use of Laboratory Animals*, and was approved by the local institutional board (Bureau de la Protection Animale). Primary cultured neurons were obtained from 14-d-old rat embryo forebrains as previously described (9, 27). When they were in the proestrus period, as shown by the observation of daily vaginal smears, Sprague Dawley female rats (R. Janvier, Le Genest-St-Isle, France) were housed together with males for 24 h, and pregnant dams were maintained in separate cages for 14 d under standard laboratory conditions on a 12 h/12 h light/dark cycle (lights on at 0600 h) with food and water available *ad libitum*.

Living embryos were excised by cesarean section performed under anesthesia with halothane. Whole embryos were placed

in culture medium previously equilibrated at 37°C and consisting of a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (50:50, ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.) supplemented with 5% inactivated FCS (Valbiotech, Paris, France). Forebrains were carefully collected, dissected free of meninges, and gently dispersed in culture medium. After centrifugation at $700 \times g$ for 10 min, the pellet was redispersed in the same medium and passed through a 46- μm pore size nylon mesh. Aliquots of the cell suspension were transferred into 35-mm Petri dishes (Falcon, Becton Dickinson, Le Pont-de-Claix, France) precoated with poly-L-lysine to obtain a final density of 10^6 cells/dish. Cultures were then placed at 37°C in a humidified atmosphere of 95% air/5% CO_2 . The following day, the culture medium was replaced with a fresh hormonally defined serum-free medium consisting of the DMEM-Ham's F12 mixture enriched with human transferrin (1 mM), bovine insulin (1 mM), putrescine (0.1 mM), progesterone (10 nM), estradiol (1 pM), and Na selenite (30 nM), and also containing fibroblast growth factor (2 ng/mL) and epidermal growth factor (10 ng/mL) (Sigma Chemical Co., St. Louis, MO, U.S.A.). After an additional 2 d, the culture medium was renewed with serum-free medium in the absence of growth factors.

Cell treatment with bilirubin and exposure to hypoxia. Six-day-old neuronal cell cultures were exposed to bilirubin for 4 d. For this purpose, a stock solution was prepared in the dark at room temperature by dissolving 5 mg bilirubin (Sigma Chemical Co.) in 0.5 mL of 0.1 M NaOH. This solution was diluted at 1:20 in distilled water to obtain a solution containing 1 mM bilirubin. After sterilization by filtration, the solution was finally diluted in serum-free culture medium to obtain a final bilirubin concentration of 0.5 μM , which has been previously shown to induce neurotoxic effects under our culture conditions, with an ED_{50} around 1 μM (15). For subsequent cell treatment, the surrounding fluid of cultured neurons was discarded by aspiration and replaced with bilirubin-containing medium, which was not renewed over the ensuing 96-h incubation period. Control cultures were processed in parallel without addition of bilirubin.

The influence of transient hypoxia was also tested after 6 d *in vitro* in bilirubin-treated and -untreated cells by transferring the culture dishes for 6 h to a humidified incubation chamber thermoregulated at 37°C and flushed by a gas mixture consisting of 95% N_2 /5% CO_2 (9, 27). Cultures were then returned for the next 96 h to normoxic atmosphere, whereas control dishes were constantly maintained under standard conditions. Reduction in oxygen delivery to the neurons was monitored by measuring oxygen pressure in samples of extracellular medium collected just before the beginning and then immediately at the end of the hypoxic insult, by means of a gas analyzer (Corning, Halstead, U.K.).

Analysis of neuronal outcome. Cell morphology was routinely assessed by phase-contrast microscopic observations by means of a Nikon Diaphot TMD (EstLab, Strasbourg, France). Cell viability was monitored at various time intervals by the spectrophotometric method using MTT, according to Hansen *et al.* (28). The assay is based on the cleavage of the tetrazolium salt by viable cells, and the reaction produces the accu-

mulation of a water-insoluble formazan salt in cultured cells. Neurons were incubated for 3 h at 37°C with MTT (500 μ g/mL, Sigma Chemical Co.), washed twice with ice-cold PBS, and lysed in DMSO, which solubilizes formazan for subsequent quantification. OD was measured at 519 nm and data were compared with those obtained from sister control cells.

Morphologic hallmarks of apoptosis and necrosis were analyzed as previously documented (15, 27, 29, 30) by staining fixed cultured cells for 10 min with the fluorescent dye DAPI (Sigma Chemical Co.) used at 0.5 μ g/mL (31). Using this procedure, healthy neurons exhibit intact round-shaped nuclei with diffuse fluorescence, indicative of homogeneous chromatin. Necrotic neurons are characterized by highly refringent smaller nuclei with uniformly dispersed chromatin, whereas condensation and fragmentation of chromatin lead to typically shrunken nuclei in apoptotic neurons. Characteristic nuclei were scored under fluorescence microscopy (Zeiss Axioscop, EstLab, Strasbourg, France) at an excitation wavelength of 365 nm in at least three separate experiments by counting concerned cells in at least three distinct areas of 100 neurons per culture dish.

Rates of protein synthesis. Time-dependent biosynthetic rates of total proteins was monitored in neuronal cells by measuring the incorporation of radiolabeled L-leucine in the acid-insoluble cellular fraction (9). Before cell exposure to bilirubin or hypoxia, the culture medium was discarded, and the cells were loaded with 2 mL of fresh serum-free medium containing 0.5 μ Ci/mL of L-[4,5- 3 H]leucine (specific activity: 1.92 TBq/mmol, New England Nuclear, Boston, MA, U.S.A.), the final concentration of the amino acid corresponding to 19.3 mM. At various time intervals, the incorporation rate of leucine was measured. Cultures were washed twice with ice-cold saline and treated for 20 min with 10% TCA at 4°C. TCA-soluble material was then collected, and the cells were washed twice with 0.5 mL ethanol. One hundred-microliter samples were taken after ethanol evaporation under air stream for radioactivity determination using a scintillation spectrometer (Beckman, model LS-1801, Strasbourg, France). The TCA-insoluble fraction remaining on the bottom of the culture dishes was solubilized with 1 mL of 1 M NaOH and aliquots were used for scintillation counting and measurement of protein concentrations by the Bradford method (32).

Glucose transport and incorporation. Functional activity of cultured neurons was evaluated by measuring specific transport and incorporation of the glucose analogue, 2-D-[3 H]deoxyglucose ([3 H]2-DG), into the cells (33). Culture dishes were washed twice in a HEPES-buffered Krebs-Ringer solution (125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM HEPES, pH 7.4) and incubated in the buffer solution for 15 min at 37°C. The medium was then discarded and the assay was initiated by adding 1.5 mL of the Krebs-Ringer medium containing 1 mM [3 H]2-DG (960 GBq/mmol, New England Nuclear). After a 30-min incubation, the cultures were rapidly washed three times with physiologic saline, dried, and solubilized in 1 mL of 1 M NaOH. Samples were taken for scintillation counting and measurement of protein contents. Nonspecific uptake of [3 H]2-DG was as-

sessed by measuring the residual radioactivity in the cultured cells by the same experimental procedure performed in the additional presence of 100 mM D-glucose.

Analysis of free radical generation. In an attempt to evaluate the influence of bilirubin on the amount of reactive oxygen species that can be generated by hypoxia, intracellular levels of free radicals were monitored in individual cells by flow cytometry using the fluorogenic dye DHR, which can easily cross cell membranes and becomes fluorescent upon oxidation by reactive oxygen species, mainly superoxide, to finally generate rhodamine 123 (34, 35). Neuronal cells were covered with 1 mL of fresh serum-free medium containing 150 μ M DHR (Molecular Probes, Eugene, OR, U.S.A.) from a 15-mM stock solution in dimethylformamide. Culture dishes were then replaced in the incubator at 37°C for 5 min, and neurons were suspended by slow horizontal agitation in the presence of 1 mL PBS containing 0.5% trypsin. The effects of trypsin were then blocked by the addition of 1 mL PBS enriched with 0.5 mM Ca²⁺/Mg²⁺, and the cell suspension was collected in a polypropylene tube stored on ice before analysis by means of a Coultronics Epics XL (Margency, France) flow cytometer equipped with an argon laser. The green intracellular fluorescence of rhodamine 123 was measured at an excitation wavelength of 488 nm by studying at least 25,000 living cells for each assay.

RESULTS

Cell viability and nuclear morphology. At 6 d *in vitro*, cells cultured from the embryonic rat forebrain showed polygonal perikarya interconnected by a dense fiber network. Immunohistochemical characterization by using antibodies raised against neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) confirmed that such preparations were highly enriched in neurons, depicting $92.5 \pm 2.8\%$ of NSE-positive cells, with $7.6 \pm 1.6\%$ of GFAP-positive cells (not shown).

Transient exposure to anaerobic gas mixture routinely reduced the P_O₂ in the culture medium by $78 \pm 3\%$, compared with control cultures maintained in normoxia. Consecutively to the hypoxic stress, neuronal alterations could be first noticed by 48 h after reoxygenation, as already reported (9, 27). At 96 h postreoxygenation, analysis of cell viability showed a 23% reduction of the number of living cells, compared with matched control neurons (Fig. 1). Nuclear staining with DAPI confirmed the higher increase in the number of apoptotic rather than necrotic neurons by the end of the experimental period (Table 1).

At the concentration of 0.5 μ M, bilirubin induced a delayed and progressive decline in the number of living neurons that finally reached 24.5% below controls at 96 h, as depicted by the MTT assay (Fig. 1). As previously reported (15), a significant number of cell nuclei stained by the fluorescent dye DAPI exhibited characteristic morphologic features of apoptosis, such as chromatin condensation. Necrotic cells could be additionally observed in bilirubin-treated cultures, but the percentage of apoptotic nuclei increased more markedly (Table 1).

Finally, when cultured cells were exposed to hypoxia in the presence of bilirubin, neuronal injury was more evident than

with either treatment taken separately (Fig. 1, Table 1). Neuronal loss reached 34%, and genomic DNA staining by DAPI revealed the presence of 23% of neurons exhibiting evident signs of apoptosis.

Protein synthesis. In control normoxic cells, radioactivity measured in the acid-precipitable fraction, which reflects biosynthetic activity, progressively increased as a function of time after cell loading with L-[³H]leucine. It varied from 2728 ± 198 to 274,450 ± 7,820 dpm/dish between 1 h and 96 h. Hypoxia-induced cell damage was accompanied by characteristic changes in the time course of [³H]leucine incorporation rates (Fig. 2). By 1 h after the onset of hypoxia and then at 48 h after reoxygenation, two distinct marked increases in protein synthesis in hypoxic neurons (+44% and +50%, respectively) were recorded. Thereafter, rates of protein synthesis declined, in parallel with the appearance of morphologic signs of cell injury. Similar temporal profiles of synthesis were recorded in cultured neurons exposed either to hypoxia in the presence of bilirubin or to bilirubin alone, although the second peak of protein synthesis was more prolonged in the latter situations (Fig. 2). These data suggest that, in all cases, neuronal damage required *de novo* protein synthesis, as previously shown in other apoptosis paradigms (36–38). This was supported by the significant protective effects of the protein synthesis inhibitor CHX in both hypoxia- and bilirubin-treated neurons. In the presence of CHX (1 μM), the final rate of viable neuronal cells was in the range of that measured in cultures exposed to CHX alone (Table 1). Moreover, whereas the number of necrotic cells recorded with CHX tended to be more elevated than in control cultures, the synthesis inhibitor abolished nucleic morphologic features of apoptosis in culture preparations exposed to hypoxia or bilirubin.

2-DG transport and incorporation and free radical generation. Control values of [³H]2-DG-specific uptake in cultured neurons were remarkably stable at the various time points studied over the experimental period. They corresponded to 112.6 ± 2.7 and 119.4 ± 1.8 nmol deoxyglucose per mg of protein at 6 h and 96 h, respectively. As shown in Figure 3, the

Table 1. Neuronal viability and nuclear morphology scored at 96 h after exposure to hypoxia, bilirubin, or both treatments—effects of CHX and glutamate receptor antagonists

	Viability score (% from controls)	Necrosis (% of total neurons)	Apoptosis (% of total neurons)
Controls	100.0 ± 6.9	5.2 ± 1.4	2.4 ± 0.8
+ 1 μM CHX	92.2 ± 4.6	9.3 ± 2.6*	3.0 ± 0.9
+ 10 μM MK-801	97.6 ± 3.5	5.8 ± 0.9	2.7 ± 0.4
+ 10 μM NBQX	96.4 ± 4.7	6.0 ± 1.2	2.9 ± 0.6
Hypoxia	77.3 ± 2.1**	9.2 ± 0.6**	13.4 ± 1.1**
+ 1 μM CHX	90.5 ± 5.1††	11.1 ± 2.2**	2.8 ± 1.0††
+ 10 μM MK-801	78.9 ± 4.4**	8.8 ± 0.6**	10.3 ± 1.7**
+ 10 μM NBQX	74.3 ± 3.0**	9.1 ± 1.1*	12.8 ± 1.3**
Bilirubin	75.5 ± 1.9**	12.0 ± 1.3**	16.2 ± 1.5**
+ 1 μM CHX	90.6 ± 4.9	13.3 ± 1.8*	2.9 ± 0.5
+ 10 μM MK-801	97.8 ± 2.3	6.9 ± 1.0	3.8 ± 0.6
+ 10 μM NBQX	76.0 ± 3.6**	14.0 ± 1.6**	14.7 ± 0.9**
Hypoxia + Bilirubin	66.0 ± 2.5**,††	16.9 ± 3.3**,††	22.6 ± 3.1**,††
+ 10 μM MK-801	88.8 ± 2.8**	8.4 ± 0.8*	8.9 ± 0.7**
+ 10 μM NBQX	69.1 ± 3.7**	17.1 ± 2.9**	20.5 ± 2.8**

Cell viability was assessed by using MTT, whereas healthy, necrotic, and apoptotic neurons were scored by fluorescence microscopy after nuclear staining by DAPI. Data are reported as means ± SD obtained from three to five separate experiments. CHX or glutamate receptor antagonists (MK-801 and NBQX) were added to the culture medium before exposure to hypoxia or/and bilirubin. Statistically significant difference *versus* the corresponding pharmacologically treated control: * *p* < 0.05 and ** *p* < 0.01; statistically significant difference *versus* hypoxia alone: †† *p* < 0.01 (Dunnett's test for multiple comparisons).

Figure 1. Effects of hypoxia or/and bilirubin on cell viability monitored as a function of time by the MTT method. Data were obtained from at least three separate experiments using 10–15 dishes per time point for each treatment, and are expressed as mean percentage of changes (± SD) compared with sister control cultures maintained in standard normoxic conditions. Statistically significant difference from controls: **p* < 0.05 and ***p* < 0.01; statistically significant difference from hypoxia alone: °°*p* < 0.01 (Dunnett's test for multiple comparisons).

Figure 2. Time course of specific changes in L-[³H]leucine incorporation rates into acid-insoluble fractions of neurons submitted to hypoxia or/and bilirubin. Data were obtained from at least three separate experiments using 10–15 dishes per time point, and are expressed as mean percentage of changes (± SD) compared with sister control cultures. Statistically significant difference from controls: ***p* < 0.01; statistically significant difference from hypoxia alone: °°*p* < 0.01 (Dunnett's test for multiple comparisons).

uptake of [³H]2-DG was significantly increased following hypoxia, and such an apparent hypermetabolism lasted for at least 24 h after reoxygenation. Thereafter, [³H]2-DG uptake progressively decreased concomitantly with the appearance of morphologic alterations, to finally reach 42% below control values at 96 h posthypoxia. By contrast, bilirubin led to a continuous, significant, and long-lasting reduction of [³H]2-DG transport and incorporation into the cultured cells. When neurons were submitted to combined treatments, [³H]2-DG

Figure 3. Time course of specific changes in specific transport and incorporation of 2-[³H]-D-deoxyglucose into cultured neuronal cells exposed to hypoxia or/and bilirubin. Data were obtained from at least three separate experiments using 10–25 dishes per time point, and are expressed as mean percentage of changes ($\pm$ SD) compared with sister control cultures. Statistically significant difference from controls: ** $p < 0.01$; statistically significant difference from hypoxia alone: ° $p < 0.05$ and °° $p < 0.01$ (Dunnett's test for multiple comparisons).

uptake was increased by the end of the hypoxic episode, as observed with hypoxia alone, but remained elevated until 72 h postreoxygenation.

Therefore, one may consider that the additional presence of bilirubin helps to maintain glucose transport and incorporation, a process necessary for energy metabolism, in hypoxic neurons. In an attempt to explain this unexpected observation, and providing that bilirubin has been suspected to be a free radical scavenger (39, 40), we analyzed the intracellular generation of free radicals in our culture model. At the concentration used, bilirubin by itself was devoid of effect on the basal radical generation monitored by the mitochondrial probe DHR (Fig. 4). Whereas the neuronal production of free radicals was transiently increased in response to hypoxia, no attenuation could be obtained in the additional presence of bilirubin.

Effects of glutamate receptor antagonists. Because earlier studies have implicated glutamate in bilirubin neurotoxicity (15, 41, 42), the influence of glutamate receptor antagonists was also tested. For this purpose, the two glutamate receptor

Figure 4. Effects of hypoxia and bilirubin on intracellular free radical production detected by flow cytometry using the fluorescent dye DHR. The data are reported as mean fluorescence units recorded at 6 h (time corresponding at the reoxygenation time) and then 24 h later in treated neurons as well as in their matched controls. Measurements were performed in two separate experiments, and the samples were run in duplicate for each time point. Statistically significant difference from controls: ** $p < 0.01$ (t test).

antagonists MK-801 (10 μ M, Tocris, Bristol, U.K.) and NBQX (10 μ M, Tocris), which are known to be selective for NMDA and non-NMDA receptors, respectively, were added to the culture medium just before exposure to bilirubin or/and hypoxia and they were then left for 6 h.

Whereas these compounds had no effects by themselves on neuronal outcome, the presence of the non-NMDA receptor antagonist NBQX did not exert any influence on the consequences of either hypoxia, bilirubin, or the combination of both treatments (Table 1). Regarding the NMDA receptor antagonist MK-801, its action varied according to the cell treatment (Table 1). When cultured neurons were subjected to hypoxia alone, no significant effect of MK-801 could be observed, whereas the compound fully protected neuronal cells against the toxic effects of bilirubin. Finally, MK-801 had only partial—but significant—protective effects on both the vital score and the percentage of apoptotic nuclei in the case of exposure to the combination hypoxia plus bilirubin.

DISCUSSION

Whereas the data reported herein confirm the recent demonstration of bilirubin-induced neuronal apoptosis in the developing brain (15), they show that the presence of low concentrations of unconjugated bilirubin, which displays a high affinity for phospholipids of neuronal cell membranes (43, 44), can potentiate cerebral damage resulting from transient hypoxia, and thus may contribute to the associated acute and subsequent long-term disturbances of the CNS (1, 2). Furthermore, cumulative toxic effects of bilirubin and hypoxia suggest the involvement of partly different mechanisms of action at the cellular level.

Following exposure to either hypoxia or bilirubin, neuronal death was dependent upon the regulation of protein synthesis. The recorded changes in the rates of protein synthesis preceding apoptotic death of cells exposed to hypoxia and/or bilirubin probably reflect selective induction or repression of specific gene products that are directly involved in the regulation of the apoptotic cascade (9, 27, 45, 46). However, the variations—both in magnitude and time—observed in leucine incorporation between the different treatments suggest a possible specificity in the genetic control of neuronal outcome according to the nature of the stress. In other words, protein synthesis triggered by bilirubin may partly differ from that triggered by hypoxia, and this might render bilirubin-treated neurons more sensitive to the hypoxic insult. It would be therefore of interest to address this question in a future study by analyzing the profile of key apoptosis-related proteins in cultured neurons exposed to bilirubin.

Changes between hypoxia- and bilirubin-treated neuronal cells were more strikingly different with regard to energy metabolism, as reflected by glucose uptake and incorporation. Although not shown in the present study, there is, under severe hypoxic conditions, a rapid failure in ATP generation, resulting in transient activation of anaerobic glycolysis. It has been demonstrated that glucose is the primary energy substrate for the brain in fetuses and newborns as well as in adults (47, 48). Although other substrates such as ketone bodies may provide

alternate fuels in some pathologic conditions, their consumption requires oxygen and, thus, only glucose is capable of sustaining brain energy metabolism in hypoxia *via* anaerobic glycolysis. Therefore, the transient increase in [^3H]deoxyglucose incorporation into cultured cells that was observed after exposure to hypoxia may reflect the activation of glycolysis *in vitro*, as previously reported in the same experimental model (33), and *in vivo* (49). Thereafter, energy metabolism progressively declined concomitantly to cell degeneration. By contrast, a significant reduction of glucose uptake was continuously recorded in bilirubin-treated neurons. Bilirubin has been reported to disrupt energy metabolism (50), and our data are in good agreement with *in vivo* studies in developing rats that have shown that hyperbilirubinemia induces a widespread decrease in cerebral metabolic rates for glucose, with prominent effects in brain areas known to preferentially accumulate bilirubin in kernicteric human babies (51), as well as in cerebral structures, the function of which is commonly altered (52). Finally, the presence of bilirubin in the culture medium of hypoxic neurons led to a metabolic profile closer to that recorded after hypoxia alone. Unexpectedly, the incorporation of deoxyglucose seemed to be preserved at 48 h and even at 72 h posthypoxia. However, the presence of bilirubin during the hypoxic insult failed to reduce the generation of reactive oxygen species. Although these results are still preliminary and remain to be confirmed, including by the analysis of radicals that may have diffused in the extracellular medium (33), they do not suggest a scavenging role for bilirubin in its apparent metabolic effects in hypoxic neurons, and the mechanism of action remains a matter of speculation.

Several lines of evidence suggest that the brain-damaging action of bilirubin requires the participation of glutamate toxicity (15, 41, 42). In this respect, our observations that MK-801, but not NBQX, is able to prevent bilirubin-induced cell injury in cultured neurons confirm that the effects of bilirubin certainly imply the activation of NMDA receptors. Conversely, cultured neurons were not rescued from hypoxia by the presence of glutamate receptor antagonists. These findings are consistent with earlier studies that demonstrated a lack of correlation between the effects of exposure to glutamate and those of transient hypoxia in immature neurons (53, 54). Although the presence of functional receptors for glutamate, including NMDA receptors, has been demonstrated in cultured neurons at the corresponding maturational stage (55), these neurons, in contrast to more mature cells, are not sensitive to toxic concentrations of either glutamate or one of its derivatives, such as NMDA, kainate, or AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionate) (54, 56). Pharmacological studies have revealed that a loss of protein kinase C activity is necessary for glutamate-mediated induction of neurotoxicity. This phenomenon appears to be an essential element of the excitotoxic death of neuronal cells, and cellular events responsible for linking glutamate-associated Ca^{2+} influx to protein kinase C inactivation are undeveloped in such immature neurons *in vitro* (53, 54, 57). Specifically in our model, we have previously shown that neurons became vulnerable to the application of glutamate when they were co-exposed to 30 nM staurosporine, a protein kinase C inhibitor, and that cells can be

rescued by glutamate receptor antagonists (54, 56). On the other hand, accumulative evidence has been provided to demonstrate that bilirubin inhibits protein kinase C activity with subsequent blockade of protein kinase C-mediated phosphorylation of endogenous substrates in various tissue systems. This process has been reported to play a role in the pathogenesis of bilirubin toxicity (58, 59). Accordingly, bilirubin, through its direct action on protein kinase C, may render the neurons sensitive to glutamate toxicity (15), and such properties may account, at least partly, for the potentiation of hypoxia effects by bilirubin.

In conclusion, whereas the toxic effects of bilirubin involve alterations of the rates of neuronal protein synthesis and energy metabolism as well as subsequent apoptotic cell death, the present study strongly suggests that low levels of unconjugated bilirubin may enhance hypoxic damage in the developing brain by allowing the additional recruitment of glutamate neurotoxicity through the activation of NMDA receptors.

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