CONTRARY TO ADULT, NEONATAL RATS SHOW PRONOUNCED BRAIN UPTAKE OF CORTICOSTEROIDS

Vikram Arya, Vincent G. Demarco, Manish Issar,1 and Günther Hochhaus

Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, Florida (V.A., M.I., G.H.); and Department of Child Health, School of Medicine, University of Missouri, Columbia, Missouri (V.G.D.)

Received September 16, 2005; accepted February 22, 2006

ABSTRACT:

Neurotoxic adverse effects after systemic corticosteroid administration are elevated in preterm infants. To test whether this might be related to an immature blood-brain barrier (BBB) that permits corticosteroids to enter the brain and induce neurotoxic effects, this study assessed the differences in brain permeability of triamcinolone acetonide after intratracheal administration to neonatal (10- to 11-day-old) and adult rats. Triamcinolone acetonide (the phosphate prodrug in the case of neonatal rats) was administered intratracheally to neonatal rats at doses of 2.5, 25, or 50 μg/kg and to adult rats at 100 μg/kg. An ex vivo receptor binding assay was used to monitor the cumulative brain and liver glucocorticoid receptor occupancies over 6 h. Brain and liver receptor occupancies in neonates were similar for the 25 and 50 μg/kg triamcinolone acetonide phosphate (brain/liver receptor occupancy ratio, 1.10 ± 0.14 and 0.87 ± 0.13, respectively), whereas some reduction in the brain permeability was seen at the lower dose. After intratracheal administration of 100 μg/kg triamcinolone acetonide to adult rats, receptor occupancies in the brain were significantly lower (brain/liver ratio, 0.21 ± 0.14; p < 0.001). The study demonstrated that glucocorticoids enter the brain of neonatal rats because of an immature BBB. The results of this study support the hypothesis that neurotoxic adverse effects in preterm infants after systemic corticosteroid administration might be related to an immature BBB.

Premature birth (defined as birth between 24–34 weeks of gestational age) continues to be a major cause of infant mortality and morbidity (Mammel et al., 1983). A majority of preterm infants are born with varying degrees of maturity of the pulmonary system. This incomplete development of the pulmonary system in premature infants mandates the use of mechanical ventilators for artificial respiratory support. The damage caused to the fragile and immature lungs by these mechanical devices predisposes the premature infant to a wide array of pulmonary disorders such as chronic lung diseases (CLD). The beneficial effects of using systemic corticosteroid therapy in the treatment and/or prevention of CLD in preterm infants have been widely documented (Georgieff et al., 1989; Groneck et al., 1995).

Despite these benefits, the administration of systemic corticosteroids to preterm infants causes increased short-term and long-term adverse effects. Several clinical studies suggested a significantly higher number of infants with cerebral palsy (O'Shea et al., 1999), reduced motor function, and somatic growth after antenatal and postnatal systemic corticosteroid therapy (Yeh et al., 1998). In a follow-up study, Yeh et al. (2004) found substantial adverse effects on neuromotor and cognitive function in school children treated with postnatal dexamethasone for CLD. Murphy et al. (2001) have shown that dexamethasone, the most widely used corticosteroid in premature infants, is associated with a significant decrease in cerebral cortical gray matter volume. As a result, concern has been voiced as to whether postnatal therapy with systemically administered dexamethasone is justified (Nicholl et al., 2002; Williams and Greenough, 2003; Halliday, 2004). Although the neurological adverse effects are widely recognized, no mechanism has been postulated for the occurrence of these neurological complications.

P-Glycoprotein (mdr1a or P-gp), an efflux transporter, is highly localized on the apical membrane of the endothelial cells of the brain capillaries (Jette et al., 1995). P-gp has the capability of effluxing a wide range of substrates including endogenous and exogenous glucocorticoids (Ueda et al., 1997; Meijer et al., 1998; Karssen et al., 2001; Uhr et al., 2002; Arya et al., 2005).

We hypothesize that significant brain levels of glucocorticoids are observed in neonatal rats after administration of glucocorticoids because of an immature BBB. These increased brain levels may result in neurotoxic adverse effects commonly observed in preterm infants, whereas levels in adults are much lower. To test our hypothesis, the receptor occupancies in the brain and liver of neonatal (10–11 days old) and adult rats after intratracheal administration of triamcinolone acetonide were monitored using an ex vivo receptor binding assay.

Materials and Methods

Preparation of Drug Formulations. Aliquots of a triamcinolone acetonide phosphate (TAP) solution (diluted to 50 μg/ml in phosphate-buffered saline; Bristol Myers Squib, Munich, Germany) were intratracheally administered at doses of 2.5, 25, and 50 μg/kg in neonatal rats. For intratracheal administration...
of triamcinolone acetonide (TA; Sigma; St. Louis, MO) in adult rats; TA powder was diluted with lactose to obtain a final concentration of 4 μg/kg.

**Animal Procedures.** All animal procedures were approved by the Institutional Animal Care and Use Committee, University of Florida. Adult Fischer rats (F-344), weighing approximately 220 to 250 g, and neonatal rats (25 ± 5 g) were obtained from Harlan (Indianapolis, IN). The rats were anesthetized with an anesthetic mixture [1.5 ml of 10% (v/v) ketamine, 1.5 ml of 2% (v/v) xylazine, and 0.5 ml of 1% (v/v) acepromazine] at a subcutaneous dose of 1 ml/kg. Once the animal was under complete anesthesia, the skin on the neck was shaved, and a 1-cm incision was made in the skin to expose the trachea. An incision was made between a pair of tracheal rings, and either TA powder (100 μg/kg to adult rats), TAP solution (2.5, 25, and 50 μg/kg to neonatal rats), or saline (to neonatal and adult rats in the placebo group) was administered via a syringe. To circumvent the problem of delivering particles of TA in the micrometer range into the relatively small lungs of neonatal rats, we decided to administer TAP in solution. The neonatal rats were kept warm with the aid of a heating pad and overhead light, and body temperature was monitored via a mouse rectal probe connected to a microprobe thermometer. The adult rats were decapitated at 0.5, 1, 2, 4, and 6 h, and the neonatal rats were decapitated at 1, 2, 4, and 6 (missing in 50 μg/kg experiments), and 6 h. Placebo animals were decapitated 3 h after dosing.

**Ex Vivo Receptor Binding Assay.** A previously developed ex vivo receptor binding assay was used to estimate the percent free receptors in the brain and liver of adult and neonatal rats. (Hochhaus et al., 1995). Immediately after removal, the tissue (brain or liver) was weighed and placed on ice followed by homogenization with 4 volumes (for brain) or 10 volumes (for liver) of ice-cold incubation buffer (10 mM Tris/HCl, 10 mM sodium molybdate, and 2 mM 1,4-dithioerythritol). Two milliliters of the homogenate was incubated with 1 ml of 5% charcoal (in ice-cold distilled water) for 10 min to remove unbound corticosteroids. After centrifugation (20 min at 20,000 g, 4°C) in a Beckman (Palo Alto, CA) centrifuge equipped with a JA-21 rotor to obtain a clear supernatant, aliquots of the supernatant (150 μl) were added to prechilled microcentrifuge tubes containing 50 μl of either 10 nM [3H]labeled TA for determining the total binding or a mixture of 10 nM [3H]labeled TA and 10 μM of unlabeled TA for determining the nonspecific binding. The microcentrifuge tubes were vortexed and incubated at 4°C for 18 h.

After 18 h, 200 μl of activated charcoal (5% in ice-cold distilled water) was added to the microcentrifuge tubes to remove excess radioactivity. The microcentrifuge tubes were vortexed, centrifuged for 5 min, and 300 μl of the supernatant was removed and added to the scintillation vial. Five milliliters of the supernatant were added to the scintillation vial. The microcentrifuge tubes were vortexed and incubated at 4°C for 18 h.

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**Results**

Control experiments performed 1, 2.5, and 6 h after intratracheal administration of placebo (saline solution) to neonatal rats showed that receptor binding data were within the experimental variability of the assay (liver, 100 ± 12%) and brain (100 ± 21%) over the observation period, with no statistical differences between the observation time points (data not shown).

Figure 1, A–C, show the percent free receptors versus time profiles in neonatal rats after intratracheal administration of 2.5, 25 and 50 μg/kg dose of TAP. Figure 1D shows the percent free receptors versus time profiles in adult rats after intratracheal administration of 100 μg/kg TA. Table 1 gives the average receptor occupancy estimates in the brain and liver and their ratios after intratracheal instillation of TAP to neonatal rats and TA to adult rats. Overall, these studies show that although the liver receptor occupancies are equivalent in neonatal and adult rats, neonatal rats at 25 and 50 μg/kg doses show significantly higher brain receptor occupancies than adult rats.

**Discussion**

We used 10- to 11-day-old nonadrenalectomized rats, as these animals reflect the physiological development of a human preterm infant (Sapolsky and Meaney, 1986). This study used a previously validated ex vivo receptor binding assay (Hochhaus et al., 1995; Suarez et al., 1998; Arya et al., 2005) to track the glucocorticoid receptor occupancy in the brain and liver of neonatal and adult rats after intratracheal administration of triamcinolone acetonide. These studies and the information given at the end of Materials and Methods ensured the validity of the approach. The major advantage of this model is that the assessment of receptor occupancies in brain and liver serves as a “surrogate marker” for the pharmacodynamically relevant free drug concentrations in different tissues. Such information is not easily obtainable from total (bound and unbound) drug concentrations. Determination of plasma and brain levels is also a challenge at these expected low drug levels.
We have previously shown that P-gp is involved in the removal of glucocorticoids from the adult rat and mouse brain (Arya et al., 2005). Our results for the 25 and 50 μg/kg doses indicate that brain receptor occupancy in neonatal rats is increased over those observed in adult animals and consequently corticosteroids are able to enter the brain. Our conclusion is that the BBB in neonatal rats is not yet fully developed and allows glucocorticoids to enter, whereas a fully developed BBB in adults prevents penetration of glucocorticoids. We therefore believe that lack of P-gp in neonatal rats might be responsible for the distinct receptor occupancy in these rats.

Matsuoka et al. (1999) studied the expression of P-gp in the brain of rats as a function of gestational age. P-gp protein was undetectable until postnatal day 7 and only reached 25% of the adult level by postnatal day 10. Normal adult levels of P-gp protein were observed at day 28. The low level of P-gp expression around day 10 (experiments were performed on postnatal day 11) is able to explain all of our results, because the limited expression of P-gp explains the inability of protecting the brain at higher doses of TA whereas the low dose (2.5 μg/kg) still does not lead to saturation of the transporter. If applicable to humans, the lack of full P-gp expression in preterm babies would also result in high levels of glucocorticoids in the brain and would explain the neurotoxic and developmental side effects observed with high doses of glucocorticoids (Halliday, 2004).

Despite the good agreement between the distinct uptake of TA into the brains of neonatal rats and the reduced expression of P-gp in these animals (Matsuoka et al., 1999), one could argue that the differences in brain receptor occupancies in the neonatal and adult rats might be due to other factors, e.g., differences in the protein binding between neonatal and adult rats or purely due to drug and formulation-related artifacts. Although slight differences in the albumin content have been described previously (Yeoh and Morgan, 1974), these differences cannot explain the pronounced differences in the brain receptor occupancy. We administered intratracheally a solution of TAP into the lung of neonatal animals whereas adult rats received a micronized TA powder. The water-soluble TAP was used to ensure that the drug reaches all areas of the neonatal lung, despite much smaller airways. To be pharmacologically active TAP needs to be activated efficiently to reach all areas of the neonatal lung, despite much smaller airways. Indeed, Seree et al. (1998) reported an induction of P-gp after administration of a very high-dose regimen of 100 mg/kg/day daily over a 5-day treatment. In contrast, other groups using lower doses (Matheny et al., 2004; Mei et al., 2004; Perloff et al., 2004) did not observe a clinically relevant induction of P-gp. We therefore believe that data presented in this communication are also relevant for the multiple dosing scenario and suggest that a potential reason for the neurotoxic effects (O’Shea et al., 1999; Yeh et al., 1998, 2004; Murphy et al., 2001; Nicholl et al., 2002; Williams and Greenough, 2003, 2004; Halliday, 2004) seen in preterm babies might be related to an undeveloped BBB.

**References**


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Address correspondence to: Dr. Guenther Hochhaus, P.O. Box 100494, Department of Pharmacutics, College of Pharmacy, JHMHC, University of Florida, Gainesville, FL 32610, E-mail: hochhaus@ufl.edu