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DMD #7419

Contrary to Adult, Neonatal Rats Show Pronounced Brain Uptake of Corticosteroids

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Running Title: Brain Uptake of Glucocorticoids in Neonatal Rats

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Abstract

Neurotoxic adverse effects after systemic corticosteroid administration are elevated in preterm infants. In order 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 (10-11 day old) and adult rats. Triamcinolone acetonide (or 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 e*x-vivo* receptor binding assay was used to monitor the cumulative brain and liver glucocorticoid receptor occupancies over 6 hours. 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), while some reduction in the brain permeability was seen at the lower dose. After intratracheal administration of 100 µg/kg of 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.

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Introduction

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 M.K et al., 1989; Groneck et al., 1993).

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 and co-workers found substantial adverse effects on neuromotor and cognitive function in school children treated with postnatal dexamethasone for CLD (Yeh et al., 2004). Murphy et al. have shown that dexamethasone, the most widely used corticosteroid in premature infants, is associated with a significant decrease in cerebral cortical grey matter volume (Murphy et al., 2001). 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). While the neurological adverse effects are

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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 due to an immature BBB. These increased brain levels may result in neurotoxic adverse effects commonly observed in preterm infants while 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 e*x vivo* receptor binding assay.

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Methods

Preparation of Drug Formulations

Aliquots of a triamcinolone acetonide phosphate (TAP) solution (Bristol Myers Squib, Munich, diluted to 50 µg/mL in phosphate buffered saline, 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) 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 (IACUC), University of Florida. Adult Fischer rats (F-344) weighing approximately 220-250 gms and neonatal rats (25 ± 5 gms) 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. In order to circumvent the problem of delivering particles of TA in the µm 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

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0.5, 1, 2, 4 and 6 hrs and the neonatal rats were decapitated at 1, 2.5, 4 (missing in 50 µg/kg experiments) and 6 hrs. Placebo animals were decapitated 3 hours after dosing.

***Ex vivo* Receptor Binding Assay**

A previously developed *ex vivo* receptor binding assay was used to estimate the % 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 vols (for brain) or 10 vols (for liver) of ice-cold incubation buffer (10 mM Tris/HCl, 10mM sodium molybdate, and 2 mM 1,4-dithioerythritol). Two mL of the homogenate was incubated with 1 ml of 5 % charcoal (in cold distilled water) for 10 minutes to remove unbound corticosteroids. After centrifugation (20 min at 20,000g, 4°C) in a Beckman centrifuge equipped with a JA-21 rotor to obtain a clear supernatant. Aliquots of the supernatant (150 µL) were added to pre-chilled microcentrifuge tubes containing 50 µL of either 10 nM ^{3}H labeled TA for determining the total binding or a mixture of 10 nM ^{3}H labeled TA and 10 µM of unlabelled TA for determining the non-specific binding. The microcentrifuge tubes were vortexed and incubated at 4°C for 18hrs.

After 18 hours, 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 minutes, and 300 µL of the supernatant was removed and added to the scintillation vial. 5 mL of the scintillation cocktail (Cytoscint™, ICN Biomed, Costa Mesa, CA) was added and the scintillation vials were read in a scintillation counter (Beckman, LS 5000 TD, Palo Alto, CA) to obtain the radioactive counts (dpm).

For each tissue (lung or liver), the area under the free receptor-time profile was calculated over the 6 hour time period ($AUC_{0\text{-}6h}$) by trapezoidal rule. $AUC_{0\text{-}6h}$ could fall anywhere between

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0 %*hr and 600 % hr indicating no receptor occupancy and full receptor occupancy respectively. The average receptor occupancy was computed by dividing the area under the bound receptor-time curve by 6 hours (duration of observation). To compensate for differences in receptor density and other factors, the ratios (average brain receptor occupancy/average liver receptor occupancy) were calculated to estimate the degree of brain uptake. The receptor occupancy data were based on three to four independent experiments (3-4 animals per time point). In order for this assay to provide differences between free glucocorticoid levels in liver and brain, the affinity of corticosteroids to the liver and brain receptors and the concentration of glucocorticoid receptors in both tissues should be comparable in neonatal and adult tissues. Turrel et al. and Csaba et al showed that the affinities of neonatal glucocorticoid receptors are similar to those of adult rats (Turrel et al., 1985; Csaba and Inczefi-Gonda, 2000). While a detailed analysis of the receptor content was not available for the neonatal rat brain, we used the specific tracer binding observed in the cytosol preparations of the placebo animals as a rough estimate of the receptor content. These experiments indicated a slight difference in the number of receptors when expressed as nMol/g of tissue (brain/liver receptors=0.5) for adult animals. Placebo data for the neonatal rats, resulted in brain level that were 30-50% of those of adults. While this indeed suggests that brain levels in neonatals are somewhat lower than in adults and lower than the liver receptor levels and as a result the number of occupied receptors would be somewhat reduced for a given drug concentration, the assay is still valid, if one wants to test the hypothesis that an increased receptor occupancy is observed in the brain of neonatal rats (a tissue that potentially contains less receptors)

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Results

Control experiments performed 1, 2.5 and 6 hours 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 $\pm$ 12%) and brain (100 $\pm$ 21%) over the observation period, with no statistical differences between the observation time points (data not shown).

Fig. 1a-c show the % free receptors *vs* time profiles in neonatal rats after intratracheal administration of 2.5, 25 and 50 µg/kg dose of TAP. Fig. 1d shows the % free receptors *vs* 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 while the liver receptor occupancies are equivalent in neonatal and adult rats, neonatal rats at 25 and 50 µg doses show significantly higher brain receptor occupancies than adult rats.

Discussion

We used 10 to 11 day old non-adrenolectomized rats, as these animals reflect the physiological development of a human preterm infant (Sapolsky RM and Meaney MJ, 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 the Method section 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.

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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, while 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*. studied the expression of p-gp in the brain of rats as a function of gestational age (Matsuoka Y et al., 1999). 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, as the limited expression of p-gp explains the inability of protecting the brain at higher doses of TA while 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 pre-term 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 Y 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. While slight differences in the albumin content have been described (Yeoh and Morgan, 1974), these differences can not 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. In order to be pharmacologically active TAP needs to be activated efficiently in the lung or systemic circulation. Phosphatases are ubiquitous in rats and

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are present in the liver as well as in the blood and muscle. Biochemical and histochemical methods showed for example at birth high phosphatase activity in both, fast and slow muscles of rats (Gutmann et al., 1976). The activation of TAP in neonatal rats was also supported by the fact that administration of TAP resulted in a distinct liver receptor occupancy that was equivalent at the higher doses to that of TA in adult animals.

While the brain uptake observed in neonatal rats after a single dose is undisputable, one might argue that this up-take is diminished when glucocorticoids are given in a multiple dosing regimen, as multiple dosing might lead to an induction of p-gp and in a reduction of brain uptake. Indeed, Seree *et al*. reported an induction of p-gp after administration of a very high dose regimen of daily100 mg/kg/d over a 5 day treatment (Seree et al., 1998). In contrast, other groups using lower doses (Matheny et al., 2004, Perloff et al., 2004, Mei 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; Murphy et al., 2001; Nicholl et al., 2002; Williams and Greenough, 2003; Halliday, 2004; Yeh et al., 2004) seen in pre-term babies might be related to an undeveloped BBB.

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Footnotes

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Figure Legend

Fig 1. Percent free receptors *vs* time profiles in the brain and liver of neonatal and adult rats after intratracheal instillation of 2.5 (panel A), 25 (panel B) and 50 µg/kg (panel C) TAP and in adult rats after 100 µg/kg (panel D) . Data points are shown as the average (±standard deviation) of 3 (2.5, 25 and 100 µg/kg) and 4 (50µg/kg) independent experiments.

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Table 1: Average receptor occupancies in the brain and liver after intratracheal instillation

			Average Receptor Occupancy (%)		Ratio
	Dose (µg/kg)	**Number of independent experiments**	**Liver**	**Brain**	**Brain vs liver**
Neonatal Rats	2.5	4	17.4 ± 13.5	–14.7 ± 11.9	n.d.
	25	3	45.3 ± 7.8	45.7 ± 9.7	1.10 ± 0.14
	50	4	50.8 ± 13	47.0 ± 9.6	0.87 ± 0.13
Adult Rats	100	3	55.3 ± 8	11.4 ± 6.7	0.21 ± 0.14

of triamcinolone acetonide phosphate (2.5, 25 and 50 µg/kg) in neonatal rats and TA (100 µg/kg) in adult rats.

n.d.: not determined

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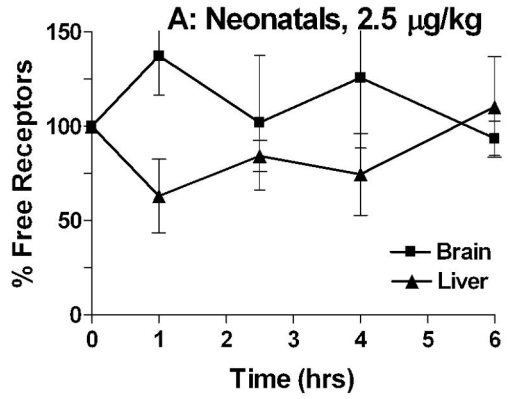
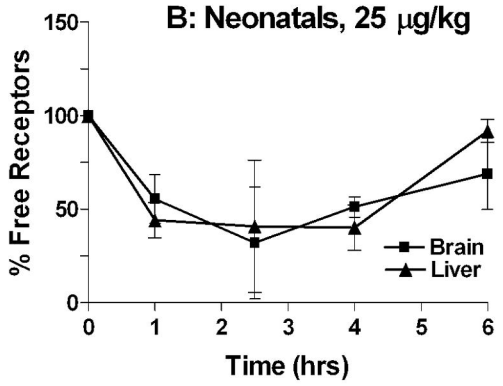
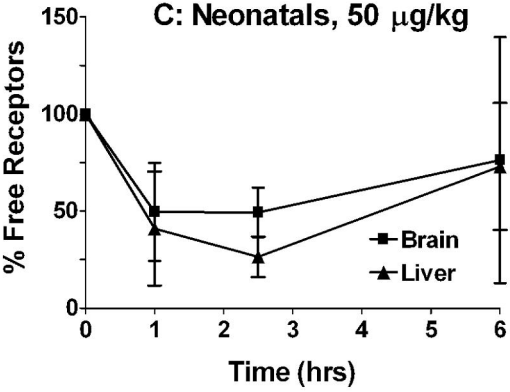

A: Neonatals, 2.5 μg/kg
% Free Receptors
150
100
50
0
0 1 2 3 4 5 6
Time (hrs)
Brain
Liver


B: Neonatals, 25 μg/kg
% Free Receptors
150
100
50
0
0 1 2 3 4 5 6
Time (hrs)
Brain
Liver


C: Neonatals, 50 μg/kg
% Free Receptors
150
100
50
0
0 1 2 3 4 5 6
Time (hrs)
Brain
Liver

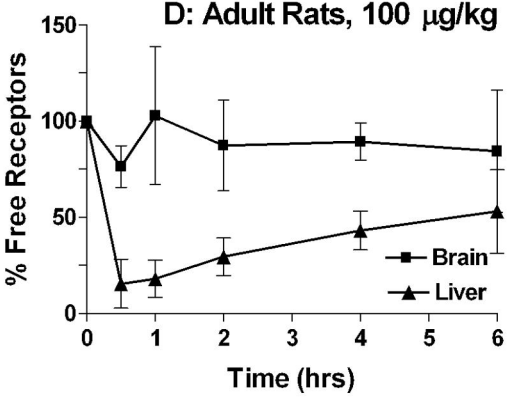

D: Adult Rats, 100 μg/kg
% Free Receptors
150
100
50
0
0 1 2 3 4 5 6
Time (hrs)
Brain
Liver

Figure 1