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Perinatal growth restriction decreases diuretic action of furosemide in adult rats

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ABSTRACT

Perinatal growth restriction programs higher risk for chronic disease during adulthood *via* morphological and physiological changes in organ systems. Perinatal growth restriction is highly correlated with a decreased nephron number, altered renal function and subsequent hypertension. We hypothesize that such renal maladaptations result in altered pharmacologic patterns for life. Maternal protein restriction during gestation and lactation was used to induce perinatal growth restriction in the current study. The diuretic response of furosemide (2 mg/kg single i.p. dose) in perinatally growth restricted rats during adulthood was investigated. Diuresis, natriuresis and renal excretion of furosemide were significantly reduced relative to controls, indicative of decreased efficacy. While a modest 12% decrease in diuresis was observed in males, females experienced 26% reduction. It is important to note that the baseline urine output and natriuresis were similar between treatment groups. The *in vitro* renal and hepatic metabolism of furosemide, the *in vivo* urinary excretion of the metabolite, and the expression of renal drug transporters were unaltered. Creatinine clearance was significantly reduced by 15% and 19% in perinatally growth restricted male and female rats, respectively. Further evidence of renal insufficiency was suggested by decreased uric acid clearance. Renal protein expression of sodium–potassium–chloride cotransporter, a pharmacodynamic target, was unaltered. In summary, perinatal growth restriction could permanently imprint pharmacokinetic processes affecting drug response.

1. Introduction

Poor nutrition during perinatal development leads to growth restriction and low birth weight, the consequences of which are both pernicious and permanent. The relationship between early life growth restriction and the predisposition for adult-onset type-2 diabetes, hypertension, and obesity has been consistently reported in human epidemiological studies and animal models (Godfrey and Barker, 2001; Luyckx and Brenner, 2005; Zandi-Nejad et al., 2006).

The higher risk for chronic disease in adults who were growth restricted at birth and postnatally stems from maladaptations, both morphological and physiological, that occur during perinatal development (Almeida et al., 2012; Barker et al., 2006). Restricted nutrient flow from mother to fetus, due to poor nutrition or placental insufficiency, prioritizes development such that organs like the brain are preserved at the expense of other organ systems (Malamitsi-Puchner et al., 2006). Of the sacrificial organs, the kidney has received the most attention. Perinatal growth restriction in humans is strongly correlated with fewer nephrons/glomeruli and reduced renal volume, a consequence of reduced nutrient delivery and reduced blood flow to kidneys during fetal development (Bagby, 2007, 2009; Ingelfinger and Schnaper, 2005).

Given their predisposition to chronic diseases, perinatally growth restricted adults are targets for pharmacological therapies; however, very little is known about the comparative effectiveness of pharmacological interventions in this population. The association between perinatal growth restriction and renal dysfunction led us to test the hypothesis that perinatal growth restriction-induced maladaptations in renal physiology could result in altered pharmacology for drugs which undergo extensive renal clearance. The loop diuretic furosemide was our drug of choice for this study

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because the kidney is its site of action, as well as its major route of elimination (Odlind and Beermann, 1980). These characteristics make urinary furosemide excretion a robust predictor of diuretic efficacy as well as an estimate of renal clearance (Nomura et al., 1981; Odlind and Beermann, 1980; Rose et al., 1976; Smith and Benet, 1979).

In the current study, we used a well-characterized rat model of perinatal growth restriction to examine the pharmacology of furosemide in adult rats that had low birth weight due to maternal protein restriction during pregnancy and lactation. We monitored the extent of diuresis, and urinary excretion of sodium, furosemide and its metabolite, furosemide-glucuronide. Furosemide is extensively plasma/serum protein bound, undergoes vectorial transport into the proximal tubule, and is renally metabolized (Lambert et al., 1982; Pichette and du Souich, 1996; Pichette et al., 1999; Smith and Benet, 1983). To characterize these potential mechanisms for variability in renal clearance, we measured *in vitro* expression of drug transporters and metabolism in hepatic and renal microsomes. We also measured creatinine and uric acid clearance as surrogate markers for altered renal physiology.

2. Materials and methods

2.1. Diets

Modified versions of the AIN76A purified diet (control, 19% protein) and the corresponding isocaloric low-protein diet (LPD, 8% protein) formulations were obtained in pellet form from Purina Test Diets (Richmond, IN). Detailed compositions of both diets are available in the literature (Cherala et al., 2006).

2.2. Experiments with dams

The study was approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University (OHSU), Portland, OR. Sprague-Dawley rats (Charles River Laboratories, Inc., Hollister, CA) were mated by housing one male rat with two virgin female rats. Day 1 of pregnancy was assigned upon observation of sperm in the daily morning vaginal smears, at which time rats were randomly assigned to one of the two diet groups. Each diet group consisted of 5–6 pregnant rats, and these rats received the assigned diet *ad libitum* throughout pregnancy and lactation.

2.3. Experiments with offspring

Upon birth, litter size and sex of pups were recorded. All litters were randomly culled to 12 pups (6 male and 6 female) on the day of birth and further randomly culled to 8 pups (4 males and 4 females) on day 4 after birth. The step-wise culling was practiced to adjust for any loss of pups during the first 72 h following birth (Cherala et al., 2006). Offspring from both groups were weaned on day 28 and were housed in isosexual groups according to perinatal diet treatment. Different dietary treatments were administered only during gestation and lactation, and all pups were weaned onto a rodent laboratory chow with access to *ad libitum* food and water.

At days 120 and 150, two male and two female offsprings from each litter were housed in separate metabolic cages with *ad libitum* food and water. At day 120, urine was collected over a 24-h period. Blood was collected at the end of 24-h period (9:00 am) the following morning, and spun at 3000*g* to collect serum within 30 min of blood collection. At day 150, between 8 am and noon, a 4-h baseline urine was collected upon intraperitoneal dosing with 1 ml/kg of vehicle (isotonic saline). The following day, a 2 mg/kg intraperitoneal dose of furosemide was administered at 8 am, followed by a 4-h urine collection. Urine outputs were measured during the same time of the day on both days in order to minimize diurnal variations (Fujimura et al., 1992). Drug and vehicle administration was carried outside of the metabolic cage. Handling of rats prior to placing on metabolic cage led to voiding of the bladder resulting in less confounding of urine output data. Similarly, at the end of urine collection window, rats were manipulated within the cage to result in the emptying of the bladder. The collection tube was protected from light to minimize photo-degradation of furosemide. The urine volumes were recorded, and an aliquot was stored at −80 °C.

About 4–5 days after furosemide administration, animals were euthanized. Prior to organ collection, blood was collected *via* cardiac puncture; serum was isolated within 30 min of blood collection and stored at −80 °C. Liver and kidneys were collected and weighed, snap-frozen in liquid nitrogen, and stored at −80 °C.

2.4. Preparation of kidney and liver samples

Crude plasma membranes were generated as previously described (Rigalli and Di Loreto, 2009). Briefly, half of whole frozen rat kidney or ~2 g of liver tissue was removed and homogenized in 12 ml of sucrose buffer (0.25 M sucrose, 0.10 M Tris, and 0.1 mM phenylmethylsulfonyl fluoride) using a dounce homogenizer. The homogenate was centrifuged at 2500*g* for 15 min at 4 °C. The supernatant was then removed and centrifuged at 24,000*g* for 20 min at 4 °C. The beige fluffy upper layer of the resulting pellet (crude plasma membranes) was resuspended in 1 ml of supernatant and stored at −80 °C. The remaining supernatant was further centrifuged at 105,000*g* for 60 min at 4 °C. The resultant microsomal pellet was resuspended in sucrose buffer and stored at −80 °C. The protein concentration of the crude plasma membranes and microsomes was measured using the Bradford method (Bradford, 1976).

2.5. Western blotting

Crude renal plasma membrane samples were subjected to Western blotting. Briefly, samples were reduced with Laemmli sample buffer (Bio-Rad, Hercules, CA) and denatured in a 100 °C water bath for 3 min. Samples were diluted to equal protein concentrations and 30 μg was loaded onto a 10× Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Hercules, CA) and subjected to electrophoresis at 250 mV for 20 min. Samples were then transferred onto a low fluorescence PVDF membrane (Bio-Rad, Hercules, CA), blocked with Odyssey Blocking Buffer (LiCor Biosciences, Lincoln, NE), and incubated with primary antibody (rabbit anti-rat antibodies; Alpha Diagnostic Inc., San Antonio, TX). The membrane was then incubated with 1:15,000 concentration IRDye® 800RD secondary antibody (LiCor Biosciences, Lincoln, NE) before being imaged on the Odyssey Imaging System (Licor Biosciences, Lincoln, NE).

To ensure proper quantification, an absorption test with a manufacturer blocking peptide (Alpha Diagnostic Inc., San Antonio, TX) was performed. Bands absorbed by the blocking peptide were quantified, using NIH ImageJ software. Recent findings from our work indicated alterations in the expression, *albeit* at mRNA level, of commonly used endogenous controls in the same samples (DuBois et al., 2013). Hence, the design of Western blotting experiments did not include an endogenous loading control. Equal loading of samples was ensured through rigorous determination of protein concentrations (in replicates) and loading of equal volumes of the same concentration, further confirmed by Ponceau S staining. Relative densitometric signal of each sample was obtained upon normalization to an assay control sample.