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Title Page

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 (2mg/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 was 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 was 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.

Keywords: fetal programming; furosemide; *in utero* growth restriction; perinatal growth restriction; pharmacokinetics; renal insufficiency.

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 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 day 120 and 150, two male and two female offspring from each litter were housed in separate metabolic cages with *ad libitum* food and water. At day 120, urine was collected over a 24-hour period. Blood was collected at the end of 24-hour period (9:00am) the following morning, and spun at 3,000 x g to collect serum within 30min of blood collection. At day 150, between 8 AM and noon, a 4-h baseline urine was collected upon intraperitoneal dosing with 1ml/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 grams of liver tissue was removed and homogenized in 12 ml of sucrose buffer (0.25 M sucrose, 0.10 M Tris, 0.1 mM phenylmethylsulfonyl fluoride) using a dounce homogenizer. The homogenate was centrifuged at 2,500 x g for 15 min at 4°C. The supernatant was then removed and centrifuged at 24,000 x 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 were 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 1:15000 concentration IRDye® 800RD secondary antibody (LiCor Biosciences, Lincoln, NE) before being imaged on 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.

2.6 Determination of *in vitro* glucuronidation of furosemide

The *in vitro* formation of furosemide glucuronide was measured in renal and hepatic microsomes, using a method adapted from Kerdpin *et al.* (Kerdpin *et al.*, 2008). Briefly,

microsomes were preincubated with alamethicin (12 µg/mg protein) on ice for 30 min. 10-100 µg of activated microsomes were then incubated with 300 µM of furosemide, 10 mM uridine 5'-diphosphoglucuronic acid trisodium salt in 100 µl of 7.4 pH 100 mM potassium phosphate buffer with 1.0 mM EDTA and 5 mM MgCl₂ at 37°C. After 90-120 min of incubation, the reaction was terminated by adding 100 µl of ice cold methanol and 3% acetic acid, vortexed, and spun at 9000 × g for 5 min. The supernatant was analyzed using high performance liquid chromatography (HPLC), as described below. The enzyme activity was reported as the amount of furosemide-glucuronide formed/min/mg of microsomal protein. Time-course experiments ensured linearity of reaction-rate and incubation with beta-glucuronidase from *Helix pomatia* identified the furosemide-glucuronide peak.

2.7 Determination of *in vitro* serum protein binding of furosemide

To determine whether there are differences in furosemide binding to serum proteins, we performed an *in vitro* binding study. Briefly, rat serum from each group was pooled and spiked with furosemide to obtain a 30 µM final concentration. The above concentration was chosen to mimic serum concentrations of furosemide in adult rats upon dosing with 2mg/kg dose (assuming 100% bioavailability). Serum was incubated at 37°C in a water bath for 10 min. An aliquot was removed to determine total furosemide. For estimation of unbound concentrations of furosemide, 0.75 ml of serum was filtered through a 30,000 molecular weight cut-off Millipore Centrifree YM-30 (Millipore Corporation, Billerica, MA) cellulose filter by centrifugation at 2,000 × g for 60 min at room temperature. Ultra filtrate obtained after centrifugation was processed and analyzed for furosemide, using HPLC method described below.

The free fraction (f_u) was determined as a ratio of unbound concentration to total concentration of furosemide. The percent increase in free fraction in the LPD group ($f_{u, LPD}$), compared to control group ($f_{u, control}$) was computed as $(f_{u, LPD} - f_{u, control}) \times 100 / f_{u, control}$.

2.8 Quantitative analysis of urinary furosemide and furosemide-glucuronide

Urine samples (100 μ l) were diluted with 100 μ l methanol and 3% acetic acid, vortexed, and spun at 9000 \times g for 5 min. The supernatant was analyzed using a HPLC method adapted from Smith *et al.* (Smith *et al.*, 1980), with a Shimadzu LC-20AD Prominence liquid chromatography on a Phenomenex 2.6 μ C18 reversed-phase Kinetex column (100 cm \times 4.60 mm), and quantitated with a Shimadzu RF-10AXL fluorescence spectrophotometer at excitation/emission wavelengths of 345/405 nm. At a flow rate of 0.9 ml/min in a solvent system of 28% acetonitrile and 30 mM H₃PO₄, furosemide and furosemide-glucuronide had retention times of 6.50 and 4.25 min, respectively. The *in vitro* incubation samples as well as serum samples were run at 35% acetonitrile and 15 mM H₃PO₄, with furosemide and furosemide-glucuronide retention times of 3.5 and 1.9 min, respectively. A standard curve of furosemide was constructed in the linear range of 10–100 μ g/ml. Inter- and intra-day variability in slopes was <10%. Equality of extinction coefficients was assumed between furosemide and the glucuronide metabolite because the glucuronic-acid cofactor did not fluoresce at 345/405 nm wavelength.

2.9 Quantitative analysis of urinary sodium

Sodium concentrations were measured using flame photometry (Cole-Parmer Instrument Company, Model 2655-10, Vernon Hills, IL).

2.10 Quantitative analysis of serum and urine creatinine and uric acid

Serum and urinary concentrations of creatinine and uric acid were determined by the simultaneous method by Zhiri *et al.* (Zhiri *et al.*, 1985). Briefly, 10 μ L of urine/plasma was diluted with 495 μ L of acetonitrile, vortexed, and spun 9000 \times g for 10 min. Ten μ L of supernatant was injected onto a 100 \times 3 mm, 2.6 μ Kinetex XB-C18 column (Phenomenex Inc, Torrance, CA), and quantitated with a Shimadzu SPD-20A UV detector at 235nm. At a flow rate of 0.5 ml/min in solvent system of 30 mM ammonium acetate and 156 mM methanol pH 7.0, uric acid and creatinine had retention times of 3.33 and 4.00 min, respectively. A standard curve for creatinine was constructed in the linear range of 12.5-400 μ g/ml, and 3.125-100 μ g/ml for uric acid. Inter- and intra-day variability in slopes was <10%.

2.11 Quantitation of albumin

Serum concentrations of albumin were determined as described by Hayashi T *et al.* (Hayashi et al., 2002). Briefly, 2 μ l of unprocessed rat serum was injected onto Asahipak ES-502N column (100 \times 7.5 mm; Phenomenex Inc, Torrance, CA) maintained at 37°C. The mobile phase consisted of buffer (0.05 M sodium acetate 0.40 M sodium sulfate (pH4.85)) and ethanol pumped at 1 ml/min. The gradient program includes ethanol concentration from 0 to 10% v/v from 0 to 50 min, followed by a return to baseline by 55 min, and 10 min of column equilibration. The eluent was monitored using a fluorescence detector at 280/340 nm excitation and emission wavelengths, respectively.

2.12 Data analysis

Data were tested for normality and equal variance, and expressed as mean \pm S.D., unless otherwise specified. Two-way ANOVA was conducted using factors [Diet (control vs. LPD) \times gender (male vs. female)] followed by Student-Newman-Keuls post-hoc test. For non-parametric data, two-way ANOVA on ranks was performed. All statistical tests were conducted at $p < 0.05$ (SigmaPlot v11.0, San Jose, CA).

All experiments include a sample size of 5-6 litters in each diet group, except for *in vitro* protein binding study where serum from all litters in a diet group were pooled to perform the experiment. Whenever data from two or more littermates were available, an average value was calculated, and considered as a single data point. The arithmetic mean \pm SD of the litters (5-6) was reported. This is the recommended method of calculating a mean for multiparous species to avoid artificial inflation of the power of statistical tests (Holson and Pearce, 1992; Zorrilla, 1997).

3. Results

3.1 Confirmation of *in utero* growth restriction (IUGR)

In the current study, both male and female offspring in the LPD group had 16% reduction in birth weight, confirming IUGR (male: 6.61 \pm 0.71 g (mean \pm S.D., control) vs. 5.53 \pm 0.42 g (LPD), $P < 0.05$; female: 6.15 \pm 0.74 g vs. 5.17 \pm 0.34 g; $P < 0.05$; Table

1). Birth weight was also significantly lower in females, compared to males, in both diet groups ($P < 0.01$). Body weight at day 150 was significantly lower for both genders in the LPD group (male: 676 ± 48 g vs. 521 ± 54 , $p < 0.001$; female: 330 ± 29 g vs. 264 ± 17 g, $P < 0.05$). Body weight at day 150 was significantly lower in females, compared to males, in both diet groups ($P < 0.01$). Absolute kidney weight was significantly lower in LPD diet group (male: 2.48 ± 0.19 g vs. 1.92 ± 0.22 ; $p < 0.001$; female: 1.28 ± 0.11 g vs. 0.98 ± 0.04 ; $P < 0.001$). However, kidney weight normalized to body weight was similar in both diet groups. Gender differences in absolute kidney weight ($P < 0.001$) were also ameliorated upon normalization to body weight. Absolute liver weight was also significantly less in LPD diet group (male: 22.22 ± 3.66 g vs. 16.76 ± 2.09 ; $P < 0.001$; female: 11.05 ± 1.19 g vs. 8.32 ± 0.35 ; $P < 0.001$). Similar to kidney, gender differences were present ($P < 0.001$), but no differences were observed in liver weight normalized to body weight in either treatment or gender groups.

3.2 Perinatal growth restriction decreases furosemide diuretic response during adulthood

In 150-day old male offspring, pre-dose (baseline) urine output was similar between diet groups (male: 5.56 ± 2.07 ml/kg of body weight, control vs. 5.74 ± 0.94 , LPD; female: 6.38 ± 3.88 ml/kg vs. 3.93 ± 1.64 ; Fig 1A). The extent of diuresis in LPD group animals, however, was decreased compared to the control group (male: 19.21 ± 2.64 ml/kg vs. 16.72 ± 1.90 , $P < 0.05$; female: 15.74 ± 3.77 ml/kg vs. 11.69 ± 1.54 ; $P < 0.05$; Fig 1B). The altered diuretic response was corroborated by urinary excretion rate of sodium. Significantly lower natriuresis was observed in LPD group offspring upon dosing with furosemide (males: 259.8 ± 61.5 μ moles/hr vs. 184.9 ± 37.6 , $p < 0.05$; females: 110.8 ± 38.2 μ moles/hr vs. 74.6 ± 15.7 , $P < 0.05$; Fig 1D). There was no difference in pre-dose sodium excretion (males: 95.94 ± 54.80 μ moles/hr vs. 97.70 ± 42.88 ; females: 57.38 ± 14.76 μ moles/hr vs. 46.65 ± 27.66 ; Fig 1C), Compared to males, females excreted significantly smaller amounts of sodium in both diet groups during pre-dose and dosed periods ($P < 0.001$).

As expected, furosemide treatment has resulted in increased urine output in male and female offspring of both diet groups ($P < 0.01$). Similarly, increased natriuresis was

observed in male and female offspring of control diet groups and male offspring of LPD group ($p < 0.05$), but not in female offspring of LPD group.

3.3 Expression of pharmacodynamic receptor is unaltered by perinatal growth restriction

To determine whether the reduced diuretic response could be explained by changes in the furosemide pharmacodynamic receptor Nkcc2, protein expression was quantified using Western blotting (Fig 2A-B). Nkcc2 expression in LPD rats was unaltered in males (0.96 ± 0.12 , control vs. 0.85 ± 0.35 , LPD) and in females (0.76 ± 0.18 vs. 0.71 ± 0.29).

3.4 Urinary excretion of furosemide is decreased in perinatally growth restricted rats

We measured urinary excretion rate of furosemide as an overall marker of renal clearance processes (Fig 2C). The percentage of the dose excreted was decreased in the LPD group (male: 61.11 ± 4.53 %dose, control vs. 51.64 ± 5.88 , LPD; $P < 0.01$; female: 47.78 ± 8.45 %dose vs. 40.58 ± 7.56 ; $P < 0.01$). Additionally, females in both diet groups excreted a significantly smaller fraction of the dose, compared to their male counterparts ($P < 0.001$).

3.5 Expression of renal drug transporters is unaltered by perinatal growth restriction

To determine whether the reduction in urinary furosemide excretion is caused by altered renal transport of furosemide, we measured the protein expression of Oat1 and Oat3 drug transporters. Molecular weights quantified for Oat1, and Oat3 were 70, and 110 kDa, respectively (Fig 3A-B). There was no perinatal diet effect on the expression of transporter proteins in either male or female rats (Fig 3C-D). The Oat1 and Oat3 expression in males, compared to females, was higher in both diet groups ($P < 0.05$).

3.6 Renal and hepatic metabolism is not increased by perinatal growth restriction

The decreased urinary excretion of furosemide could be a result of increased furosemide metabolism. The LPD did not alter the *in vitro* rate of formation of furosemide-glucuronide in renal microsomes (male: 172 ± 27 pmoles/min/mg of

microsomal protein, control, vs. 179 ± 16 , LPD; female: 86 ± 23 pmoles/min/mg vs. 69 ± 18 , Fig 4A). Significantly slower renal *in vitro* metabolism was observed in females of both diet groups compared to males ($P < 0.001$). The hepatic *in vitro* rate of formation of furosemide-glucuronide was significantly decreased in LPD males but unchanged in LPD females (male: 119 ± 15 pmoles/min/mg, control vs. 87 ± 13 , LPD, $P < 0.05$; female: 83 ± 32 pmoles/min/mg vs. 102 ± 9 ; Fig 4B). Both control- and LPD-group animals excreted similar amounts of metabolite in urine, normalized to the total furosemide dose, in each gender (male: $0.78 \pm 0.35\%$ vs. $1.19 \pm 0.66\%$; female: $1.11 \pm 0.38\%$ vs. $0.75 \pm 0.23\%$; Fig 4C). There were no gender differences in either group.

3.7 Serum protein binding of furosemide is increased by perinatal growth restriction

The serum protein binding of furosemide was estimated in 150-day old rats in a pooled serum sample (data not shown). Enough serum was available to perform measurement in singlicate in isosexual animals of each diet group. The percent increase in free fraction, compared to control rats, was 18.6% and 7.1% in male and female rats of the LPD group, respectively. The male rats in each diet group exhibited 20% higher free fraction than their female counterparts. Free-fraction estimates of furosemide in the serum of male rats were 0.0041 (control) and 0.0049 (LPD), and in female rats were 0.0033 (control) and 0.0035 (LPD). The changes in free fraction were noted in the absence of alterations in serum albumin concentrations (data not shown).

3.8 Creatinine and uric acid clearance are decreased in perinatally growth restricted rats

We estimated creatinine and uric acid clearance as surrogate markers of renal function (Fig 5A-B). The estimated creatinine clearance was decreased in LPD group (male: 8.22 ± 1.75 mL/min, control, vs. 6.95 ± 1.51 , LPD, $P < 0.05$; female: 4.22 ± 0.63 mL/min vs. 3.42 ± 0.25 ; $P < 0.05$). Females in both diet groups had significantly lower creatinine clearance compared to males ($P < 0.001$). Similarly, uric acid clearance was decreased in LPD group (male: 0.41 ± 0.11 mL/min, control, vs. 0.24 ± 0.09 , LPD, $P < 0.001$; female: 0.19 ± 0.02 vs. 0.12 ± 0.04 ; $P < 0.001$). Additionally, females in both diet groups had less uric acid clearance compared to males ($P < 0.001$).

4. Discussion

An adverse intrauterine environment leading to growth restriction is robustly associated with a higher risk of chronic diseases during adulthood. Pharmaceutical interventions are a vital mode of treatment and management of these diseases; however, the efficacy of drug treatment in adults who were *in utero* as well as perinatally growth restricted is poorly understood. In the current study using perinatally growth restricted rats, we found that the diuretic efficacy of furosemide is altered due to programming of molecular pharmacokinetic processes.

In growth restricted rats, furosemide-induced diuresis and natriuresis were decreased despite similar baseline urine outputs. Previous reports indicate that furosemide-induced diuresis and natriuresis are best explained by urinary furosemide excretion, instead of plasma concentrations (Nomura et al., 1981; Odland and Beermann, 1980; Rose et al., 1976; Smith and Benet, 1979); hence, we compared urinary excretion. The diminished pharmacological response of furosemide was corroborated by reduced urinary excretion of furosemide, suggesting that altered pharmacokinetic processes such as drug transport and metabolism may be at play.

Furosemide is actively secreted across the basolateral membrane by Oat 1 and 3 (Vallon et al., 2008). As reported by others (Ljubojevic et al., 2004), we also observed a male-predominant expression of Oat1 which would correlate with the higher urinary excretion rate of furosemide in males in both diet groups. This is further corroborated by other reports in female rats of decreased renal clearance of furosemide and para aminohippuric acid (a prototypical Oat substrate) compared to males (Cerrutti et al., 2002). However, there were no differences in transporter expression in growth restricted rats, indicating that the altered urinary excretion of furosemide may be explained by mechanisms other than renal transport.

The predominant furosemide metabolite in humans and rats is furosemide-glucuronide, mediated by uridine 5'-diphospho-glucuronosyltransferase. It has been observed that approximately two-thirds of the metabolic clearance is attributable to

kidney, and the remainder in the liver (Wallin et al., 1977). Similarly, while the majority of the systemic clearance (sum of metabolic and non-metabolic clearance) of furosemide occurs in the kidney, the liver does contribute; in the rat, hepatic clearance of furosemide has been estimated to account for 20% of systemic clearance (Prandota and Pruitt, 1991), and in rats variability in non-renal metabolism can account for variability in the diuresis (Lambert et al., 1982). In growth restricted rats, there were no differences in either *in vivo* urinary excretion of furosemide-glucuronide or *in vitro* formation of furosemide-glucuronide in renal microsomes. This would suggest that renal metabolism would not explain the difference in urinary furosemide excretion. Given the relative contribution of liver metabolism, we also measured *in vitro* metabolism of furosemide in hepatic microsomes. Curiously, hepatic microsomes from growth restricted males showed a ~27% decrease in the rate of furosemide-glucuronide formation; in females, there were no differences between growth restricted and control, and no gender difference was observed. These trends would not suggest that the decreased urinary excretion of furosemide is explainable by intrinsic hepatic metabolism.

Lastly, although we did not measure nephron number, multiple reports have indicated up to ~30% reduction in the nephron number in a similar animal model (Luyckx and Brenner, 2005). If the concentration of drug transporters and drug metabolizing enzymes in the kidney are unchanged, what could be rate-limiting for urinary excretion is a decrease in the number of nephrons and/or a decrease in renal sufficiency. Creatinine clearance is a common surrogate marker for renal filtration, and uric acid is a less robust but still relevant marker for renal tubular dysfunction (Louthrenoo et al., 2002; Ochiai et al., 2005; So and Thorens, 2010). We observed in this study significant decreases in both creatinine clearance and uric acid clearance. While the magnitude of these differences in clearance and serum values would not be interpreted to suggest renal disease, they are suggestive of an overall decrease in the renal function. In this study, perinatally growth restricted rats may not have diseased kidneys but perhaps less efficient kidneys, and of the mechanisms we studied this could be an explanation for the observed decrease in urinary excretion of furosemide.

Additionally, furosemide has been shown to induce vasodilation and reduction of cardiac output (Dormans et al., 1996). Although untested in the current study, could further potentially explain the renal changes post drug administration.

Other sources for this variability in urinary excretion were not examined in the current study. Significant drug loss as a result of first-pass metabolism is reported with intraperitoneal route of administration (Lambert et al., 1982). Previous studies and the current study noted 40-60% of drug recovery in urine suggesting ~0.5 bioavailability of furosemide upon dosing intraperitoneally (Lambert et al., 1982). However, differences in bioavailable fraction between the two groups could possibly explain the diminished furosemide response in perinatally growth restricted rats. While lack of serum data to construct a pharmacokinetic profile is typically a limitation for most drugs, the serum pharmacokinetic profile is less informative in the case of furosemide. On the other hand, serum protein binding data is demonstrated to be highly valuable in predicting furosemide delivery to renal tubules (Nomura et al., 1981; Odlind and Beermann, 1980; Rose et al., 1976; Smith and Benet, 1979). Hence, we have obtained preliminary data on serum protein binding of furosemide *in vitro* that show in perinatally growth restricted males and females an 18% and 7% increase in free-fraction of furosemide compared to their respective controls; serum albumin levels being similar between control and LPD groups (data not shown). Serum protein binding of drugs has been shown to be decreased by hyperglycemia (Koyama et al., 1997) and hyperlipidemia (Anger and Piquette-Miller, 2010), both of which are often observed in IUGR human subjects. While this data is purely speculative and not subject to statistical interrogation (experiment carried out in a pooled serum sample; n=1), growth restricted rats may have an increase in non-renal clearance given that bound-furosemide is more likely to be secreted into kidneys and escape extra-renal distribution and metabolism (Pichette et al., 1999). Further studies with urinary and serum concentration data are required to thoroughly understand the influence of serum protein binding alterations on the pharmacokinetics of furosemide, especially the degree to which renal and non-renal (hepatic) clearances are affected.

The above discussed alterations in pharmacokinetic processes of furosemide were observed in the absence of any significant changes in the renal expression of the Nkcc2 receptor protein, the pharmacodynamic receptor of furosemide. The lack of change in Nkcc2 observation in this study is in disagreement with increased expression reported in other studies (Alwasel and Ashton, 2009; Dagan et al., 2009), which could be attributable to differences in duration and extent of protein deprivation, age and strain of the animals, and tissue preparation. In the current study, a 2mg/kg dose was chosen for examining the programming effect. Upon considering 65% bioavailability of furosemide in humans (Ogata et al., 1985), this dose would translate into a human equivalent dose of 80mg which is typically the highest dose prescribed to control edema. We speculate that the diuretic and saluretic responses would be further depressed at lower doses in growth restricted subjects, giving rise to larger inter individual variability in furosemide treatment at lower range of doses. Hence, the authors plan to undertake dose response studies to confirm this speculation as well as understand the potency (parallel shift) and/or activity (changes in maximal response) of furosemide.

In summary, this study implicates perinatal growth restriction in drug efficacy during adult life, and adds to the growing list of drugs, currently hexobarbital (Cherala et al., 2007) and indomethacin (Barreto et al., 2012), with pharmacological responses shown to be programmed *in utero*. Lack of programming of pharmacodynamic processes (Nkcc2 expression) underscores the relevance of pharmacokinetic processes to develop varied strategies to personalize medicine in subjects that are growth restricted in their early life. Additional experimentation is needed to identify and characterize molecular and biochemical mechanisms underlying the fetal programming of pharmacokinetics. The clinical relevance of the current study finding is unclear, as the reduction in diuretic response was small. However, mechanistic insights into inter-individual variability of drug response are very valuable and the current study addresses sources of such variability. Furthermore, it is important to consider that birth weight, a marker of IUGR, is an easily available and highly economical (no cost) patient specific information that could contribute significantly towards optimization of pharmacotherapies.

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7. Figure Legends

Figure 1. Perinatal program of diuretic response. Male and female offspring (150 days old) of dams fed either control (closed bars) or low protein (open bars) diets throughout gestation and lactation were administered furosemide (2 mg/kg i.p. dose) at 8 AM. The 4-h urine output was adjusted for body weight (Fig 1A-B). Baseline (pre-dose) urine output was determined the previous day during the same time period (8 AM-noon; Fig 1A). Urinary excretion rate of sodium during pre-dose (Fig 1C) and upon treatment with furosemide (Fig 1D) was also measured. Each bar represents arithmetic mean \pm S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) \times gender (male vs. female)). Multiple comparisons were tested using Student-Newman-Keuls post-hoc test. * denotes significant differences between control and LPD groups ($P < 0.05$). # denotes significant gender differences ($P < 0.05$).

Figure 2. Nkcc2 receptor expression and urinary excretion of furosemide.

Representative Western blots of Nkcc2 protein in renal crude plasma membranes of 150-day old rats is shown in panel (A). Alternating control and LPD representative samples are shown for male and female rats, followed by representative section of ponceau stain. Expression of Nkcc2 was detected at 150 kDa, and results were normalized to an assay control (adult renal sample) run simultaneously with samples on all blots to account for blot variability. Relative expression of Nkcc2 (B) and urinary excretion of furosemide (C) in male and female offspring of dams fed either control diet (closed bars) or LPD (open bars) throughout gestation and lactation is shown. Each bar represents arithmetic mean \pm S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) \times gender (male vs. female)) followed by Student-Newman-Keuls post-hoc test. # denotes significant gender differences ($P < 0.05$). * denotes significant differences between control and LPD groups ($P < 0.05$).

Figure 3. Expression of renal drug transporter proteins in 150-day old rats.

Western blots of Oat1, and Oat3 proteins in renal crude plasma membranes is shown in

top panels (Oat1 (A); and Oat3 (B)). Alternating control and LPD representative samples are shown for male and female rats, followed by representative section of ponceau stain. Expression results were normalized to an assay control (adult renal sample) run simultaneously with samples on all blots to account for blot variability. Relative expression of Oat1 (C), and Oat3 (D) in male and female offspring of dams fed either control (closed bars) or LPD (open bars) diets throughout gestation and lactation is shown. Each bar represents mean \pm S.D. of 5-6 litters. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) \times gender (male vs. female)) followed by Student-Newman-Keuls posthoc test. # denotes significant gender differences ($P < 0.05$).

Figure 4. *In vitro* and *in vivo* metabolism of furosemide. The *in vitro* renal (A) and hepatic (B) metabolism of furosemide and *in vivo* renal excretion of furosemide metabolite (C) was determined in 150-day old male and female offspring of dams fed either control (closed bars) or low protein (open bars) diets throughout gestation and lactation. The *in vitro* rate of furosemide metabolism was reported as pmoles of metabolite formed /min /mg of microsomal protein. The urinary excretion of the metabolite was computed as a percent of furosemide dose administered. Each bar represents arithmetic mean \pm S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) \times gender (male vs. female)) followed by Student-Newman-Keuls post-hoc test. # denotes significant gender differences ($P < 0.05$). * denotes significant differences between control and LPD groups ($P < 0.05$).

Figure 5. Creatinine and uric acid clearance. Creatinine (A) and uric acid (B) clearance were estimated from serum and urinary concentrations from a 24-hour urine collection in day120 old rats. Each bar represents arithmetic mean \pm S.D. of 5-6 litters. Whenever data from two or more littermates were available, an average value was calculated and reported as a single data. Two-way ANOVA was conducted using factors (Diet (control vs. LPD) \times gender (male vs. female)) followed by Student-Newman-Keuls post-hoc test. # denotes significant gender differences ($P < 0.001$). * denotes significant differences between control and LPD groups ($P < 0.05$).

Table 1. Birth, body, and organ weights of day 150 old male and female offspring of dams fed either control or LPD during gestation and lactation.

	<u>Males</u>		<u>Females</u>	
	Control	LPD	Control	LPD
Birth Weight (g)	6.61±0.71	5.53±0.42 ^a	6.15±0.74 ^b	5.17±0.34 ^{a,b}
Body Weight (g)	676±48	521±54 ^a	330±29 ^b	264±17 ^{a,b}
Kidney Weight (g)	2.48±0.19	1.92±0.22 ^a	1.28±0.11 ^b	0.98±0.04 ^{a,b}
Kidney Weight (g)/ Body Weight (kg)	3.67±0.22	3.68±0.09	3.89±0.15	3.70±0.25
Liver weight (g)	22.22 ± 3.66	16.76 ± 2.09 ^a	11.05 ± 1.19 ^b	8.32 ± 0.35 ^{a,b}
Liver weight (g)/ Body Weight (kg)	3.28 ± 0.44	3.22 ± 0.28	3.34 ± 0.27	3.16 ± 0.15

^a denotes significant difference from control diet within the gender; p<0.05.

^b denotes significant difference from males within the diet group; p<0.05.