TRANSLATIONAL PHYSIOLOGY

Indomethacin administered early in the postnatal period results in reduced glomerular number in the adult rat

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Kent AL, Koina ME, Gubhaju L, Cullen-McEwen LA, Bertram JF, Lynnhtun J, Shadbolt B, Falk MC, Dahlstrom JE. Indomethacin administered early in the postnatal period results in reduced glomerular number in the adult rat. *Am J Physiol Renal Physiol* 307: F1105–F1110, 2014. First published September 3, 2014; doi:10.1152/ajprenal.00328.2014.—Indomethacin and ibuprofen are administered to close a patent ductus arteriosus (PDA) during active glomerulogenesis. Light and electron microscopic glomerular changes with no change in glomerular number were seen following indomethacin and ibuprofen treatment during glomerulogenesis at 14 days after birth in a neonatal rat model. This present study aimed to determine whether longstanding renal structural changes are present at 30 days and 6 mo (equivalent to human adulthood). Rat pups were administered indomethacin or ibuprofen antenatally on days 18–20 (0.5 mg·kg⁻¹·dose⁻¹ indomethacin; 10 mg·kg⁻¹·dose⁻¹ ibuprofen) or postnatally intraperitoneally from day 1 to 3 or day 1 to 5 (0.2 mg·kg⁻¹·dose⁻¹ indomethacin; 10 mg·kg⁻¹·dose⁻¹ ibuprofen). Control groups received no treatment or normal saline intraperitoneally. Pups were killed at 30 days of age and 6 mo of age. Tissue blocks from right kidneys were prepared for light and electron microscopic examination, while total glomerular number was determined in left kidneys using unbiased stereology. Eight pups were included in each group from 14 maternal rats. At 30 days and 6 mo, there were persistent electron microscopy abnormalities of the glomerular basement membrane in those receiving postnatal indomethacin and ibuprofen. There were no significant light microscopy findings at 30 days or 6 mo. At 6 mo, there were significantly fewer glomeruli in those receiving postnatal indomethacin but not ibuprofen ($P = 0.003$). In conclusion, indomethacin administered during glomerulogenesis appears to reduce the number of glomeruli in adulthood. Alternative options for closing a PDA should be considered including ibuprofen as well as emerging therapies such as paracetamol.

kidney; glomerular number; indomethacin; ibuprofen; premature neonate; fetus

INDOMETHACIN MAY BE ADMINISTERED to a pregnant woman as a tocolytic to prevent preterm delivery. Adverse effects of indomethacin on the fetus and neonate have been described including oligohydramnios, persistent renal insufficiency, gastrointestinal bleeding and perforation, and persistent pulmonary hypertension (8, 11, 21, 54, 58). It has not been determined

whether there is any long-term effect on fetal glomerular development or glomerular endowment following exposure to intrauterine indomethacin.

Indomethacin and ibuprofen have been shown to close a patent ductus arteriosus (PDA). The timing of administration of these medications is during a time of active glomerulogenesis (15, 16, 41, 59). Indomethacin is known to be nephrotoxic causing acute renal failure in 25% of premature neonates treated for a PDA (2). Ibuprofen is felt to be less renal toxic than indomethacin, but it is as efficacious as indomethacin at closing the patent ductus (45). Two animal studies have shown an effect on glomerulogenesis (induction of new nephrons and glomeruli) in the rat and mouse with ibuprofen and a cyclooxygenase-2 (COX-2)-selective inhibitor. In both studies, the number and size of glomeruli were decreased (3, 34). Administration of indomethacin, ibuprofen, and gentamicin to neonatal rats has shown extensive glomerular injury on electron microscopic examination with both antenatal and postnatal administration at day 14 (32). These findings are very different from those found in both adult rat and human renal biopsy findings, which show a predominantly tubular injury (4, 23, 33, 46, 47). Indomethacin was shown to have more significant suppressive effects than ibuprofen on renal COX-2 and vasodilator prostanoids in a neonatal rat model (26). Ibuprofen has been shown in premature neonates to result in a low glomerular filtration rate for a month following administration (60). A recent study has shown increased numbers of podocytes in the urine of preterm neonates receiving indomethacin, suggesting drug-induced glomerular injury at the time of ongoing glomerulogenesis (31).

In the human, glomerulogenesis is complete at 34–36 wk gestation. The metanephros begins development at 5 wk gestation, with vesicular glomerular development occurring at 18 wk gestation. Glomerular tubular development occurs from 24 wk gestation and is complete at 36 wk (1, 27, 42). In the rat, glomerulogenesis continues after birth for ~1 wk (36). Accelerated nephrogenesis and abnormal glomerular morphology have been noted in the premature human neonatal kidney (52). A decreased number of glomeruli has been implicated in the development of hypertension and subsequent cardiovascular disease in animal models and human studies (5, 7, 10, 28–30, 37, 49, 61). The implications for long-term renal and cardiovascular health in extremely premature survivors are important.

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Table 1. Control and experimental groups route, timing, and dose of medication

Group	Route	Timing	Drug and Dose
1 A	Nil	Nil	Nil
1 B	Intraperitoneal	Postnatal days 1–5	Normal saline
2	Oral	Antenatal days 18–20	Indomethacin 0.5 mg/kg
3	Oral	Antenatal days 18–20	Ibuprofen 10 mg/kg
4	Intraperitoneal	Postnatal days 1–3	Indomethacin 0.2 mg/kg
5	Intraperitoneal	Postnatal days 1–5	Indomethacin 0.2 mg/kg
6	Intraperitoneal	Postnatal days 1–3	Ibuprofen 10 mg/kg
7	Intraperitoneal	Postnatal days 1–5	Ibuprofen 10 mg/kg

At birth, the neonatal rat kidney is similar to that of a 24-wk gestation human fetus allowing administration of indomethacin and ibuprofen to the pregnant dam and the neonatal rat at similar gestations that would be given to the human fetus and extremely premature infant. This study aimed to determine whether administration of antenatal and postnatal indomethacin and ibuprofen is associated with prolonged glomerular changes at 30 days and 6 mo of age (equivalent to adulthood).

METHODS

Sprague-Dawley pregnant dams were obtained from the Australian National University animal house. The animal experiments were approved by the Australian National University Animal Research Ethics Committee and treatment and care of the animals conformed to the National Health and Medical Research Council of Australia Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Control animals consisted of pregnant dams and their offspring having no drug treatment during pregnancy or their pups receiving intraperitoneal saline. Seven groups of experimental animals were established and the method of administration, timing, and dose of medication are detailed in Table 1. Animals were killed at 30 days and 6 mo of age. Antenatal indomethacin and ibuprofen were administered during the time period that would be equivalent to indomethacin use as a tocolytic. The doses administered were those of standard oral dosing for these medications. The postnatal doses given intraperitoneally are those that are used for intravenous administration in neonates. The length of timing of the doses was equivalent to two courses of indomethacin or ibuprofen which is a common clinical scenario. Rat litters had 12–14 pups per litter with one to two early deaths in each litter. Four male and four female pups were included from each litter.

Immediately after the pups were killed, the abdominal and chest organs were exposed using a midline incision. A butterfly needle was inserted and clamped into the left ventricle and an incision was made into the right atrium. To prevent clotting, 1 U/g body wt heparin sodium (Astra Zeneca) was instilled into the circulation. To dilate the vasculature, 1.2-mg dose papaverine hydrochloride (David Bull Laboratories) was administered. The vasculature was then cleared with 0.9% sodium chloride and then perfusion-fixed with 2.5% glutaraldehyde (ProSciTech) in 0.1 M phosphate buffer. The right and left

kidneys were then excised. The right kidney was placed in 2.5% glutaraldehyde while the left kidney was placed in 10% buffered formalin.

Light microscopy. Following fixation in 2.5% glutaraldehyde for 5 h, the right kidney tissue was transferred into 10% buffered formalin for 24 h before being processed and embedded into paraffin for routine histological assessment. Two- and four-micrometer sections were cut from each sample and stained with hematoxylin and eosin, periodic acid Schiff with methenamine silver (PASM), and trichrome to better assess the basement membranes and capillary loops. The following features were assessed: intercellular edema and inflammation, cellularity, evidence of tubular damage (such as vacuolization of the epithelium, damage to the brush border, dilation of the tubules, and mitotic activity), vascular changes, and presence of calcification in the tubules or interstitium. Glomerular number was assessed using a stereological technique described below and glomerular size was assessed by electron microscopy (EM).

Glomerular structure and number. The left kidney was processed whole for embedding in glycolmethacrylate (Technovit 7100; Heraeus Kulzer), exhaustively sectioned at 15 μ m, and stained with PAS. The same technician (LG) performed the glomerular structure and counting assessment and was blinded to treatment groups. An unbiased stereological technique was used known as the physical disector/fractanator combination, previously described (6, 7).

EM. Kidneys were fixed in 2.5% glutaraldehyde (ProSciTech) for 5 h and then postfixed with 0.1 mol/l cacodylate-buffered 2% osmium tetroxide (ProSciTech) for 2 h. En bloc staining with 2% aqueous uranyl acetate (ProSciTech) preceded dehydration through a graded series of ethanol solutions. Specimens were infiltrated with Spurr's resin and set overnight at 70°C. Three levels of thin sections (100 nm) were cut from three blocks of each kidney with a minimum of two rats from each group examined. Thin sections mounted on copper/palladium grids were stained with Reynold's lead citrate and viewed on a JEOL 1011 transmission electron microscope. Images were captured using a MegaView G2 digital camera and jTEM software. The following features were assessed: percentage foot process effacement, glomerular measurement (microns), deposits, mesangial expansion, humps/bumps/splits in the glomerular basement membrane, tubule lumen dilatation, tubule mitoses, proximal tubule vacuoles, surface bleb dropouts, and alternate wedge-stripped proximal tubular degeneration.

Statistical analysis. Data were stored and analyzed using the IBM SPSS Statistics Release 22. (IBM, Chicago, IL). For numerical data, repeated-measures ANOVA adjusting for sex with Bonferroni post hoc adjustment was used. Chi square analyses were performed for categorical variables. For glomerular number, a mixed generalized linear model using a Tweedie model type with log link was used with the Chi square statistics based on a likelihood ratio method. A probability (*P*) value of <0.05 was considered statistically significant. Values are means $\pm$ SE.

RESULTS

Eight animals per group were killed at 30 days and 6 mo of age with equal numbers of males and females per group.

Table 2. Weight changes in grams of rat pups from day 1 to day 4, day 7, day 30, and 6 mo and drug treatment

Weight Difference from Day 1 g	Control	Antenatal Indomethacin	<i>P</i> Value	Antenatal Ibuprofen	<i>P</i> Value	Postnatal Indomethacin Days 1–3	<i>P</i> Value	Postnatal Indomethacin Days 1–5	<i>P</i> Value	Postnatal Ibuprofen Days 1–3	<i>P</i> Value	Postnatal Ibuprofen Days 1–5	<i>P</i> Value
Day 1 (SD)	7.1 (0.6)	6.9 (0.6)	0.38	7.8 (0.5)	0.003	7.3 (0.9)	0.66	7.0 (0.4)	0.49	7.44 (0.8)	0.23	6.8 (0.5)	0.06
Day 4	3.9 (0.8)	3.8 (0.5)	0.41	4.3 (0.7)	0.16	3.6 (0.5)	0.12	3.1 (1.5)	0.05	4.3 (0.7)	0.16	2.9 (1.0)	0.003
Day 7	10.1 (0.7)	9.9 (1.3)	0.62	9.8 (1.1)	0.43	9.2 (1.0)	0.006	8.1 (1.7)	<0.0001	9.9 (1.09)	0.56	7.0 (2.3)	<0.0001
Day 30	89.1 (5.3)	85.8 (8.2)	0.18	91.4 (6.9)	0.28	112.3 (11.4)	<0.0001	102.9 (9.4)	<0.0001	116.1 (14.4)	<0.0001	105.8 (14.5)	<0.0001
6 mo	437.0 (163.5)	459.0 (148.0)	0.78	461.3 (161.9)	0.77	521.1 (150.1)	0.3	429.8 (160.8)	0.93	496.3 (149.5)	0.46	520.6 (204.3)	0.38

Table 3. Weight of left kidneys in grams at day 30 and 6 mo of age

Weight Left Kidney g	Control	Antenatal Indomethacin	P Value	Antenatal Ibuprofen	P Value	Postnatal Indomethacin Days 1–3	P Value	Postnatal Indomethacin Days 1–5	P Value	Postnatal Ibuprofen Days 1–3	P Value	Postnatal Ibuprofen Days 1–5	P Value
Day 30 (SD)	0.66 (0.052)	0.64 (0.055)	0.52	0.65 (0.052)	0.89	0.82 (0.055)	<0.0001	0.75 (0.033)	0.001	0.88 (0.04)	<0.0001	0.78 (0.034)	<0.0001
6 mo (SD)	1.97 (0.75)	2.03 (0.7)	0.87	1.85 (0.64)	0.74	2.2 (0.63)	0.51	1.96 (0.7)	0.98	2.31 (0.83)	<0.0001	2.18 (0.7)	0.57

Weight change of rats from day 1 to day 4, day 7, day 30, and 6 mo. Rat pups receiving ibuprofen antenatally were heavier on day 1 than all other groups ($P = 0.003$). There was no significant difference with change in weight of treated pups from day 1 to day 4 compared with controls in all groups except for postnatal ibuprofen ($P = 0.003$). On day 7 rats receiving postnatal indomethacin days 1 to 3 and days 1 to 5 and ibuprofen days 1 to 5 had significantly higher weight change from day 1 ($P = 0.006$; $P < 0.0001$; $P < 0.0001$). On day 30 the weight change from day 1 in all animals receiving both antenatal and postnatal indomethacin and ibuprofen was significantly more than compared with controls ($P < 0.0001$); however, at 6 mo of age there were no differences in weight in any group compared with control (Table 2).

Weight of kidneys at day 30 and 6 mo. There were no differences in the weight of left kidneys of those rats receiving antenatal indomethacin or ibuprofen compared with controls at 30 days of age; however, left kidneys were heavier in all groups receiving postnatal indomethacin and ibuprofen at day 30 ($P < 0.0001$). This difference was no longer present at 6 mo of age (Table 3). These findings were similar for the right kidney (data not shown).

EM findings. At 30 days, the glomerular measurements were significantly smaller (measuring 90–120 μm) in those receiving postnatal indomethacin and ibuprofen ($P = 0.001$), but at 6 mo this difference was no longer evident ($P = 0.08$). At 30 days, there was no significant difference in foot process effacement ($P = 0.4$), but at 6 mo those receiving antenatal indomethacin and postnatal indomethacin had more foot process effacement (20–40%; $P < 0.0001$; Fig. 1). At 30 days, there was no difference in deposits present ($P = 0.05$), while at 6 mo there were significantly more deposits in those receiving ante-

natal indomethacin and ibuprofen and postnatal ibuprofen ($P < 0.0001$). At 30 days, there was more moderate to severe mesangial expansion in those receiving postnatal indomethacin and at 6 mo those who had received antenatal indomethacin, postnatal indomethacin, and postnatal ibuprofen ($P < 0.0001$). At 30 days, there were significantly more glomerular basement membrane humps, bumps, and splits after antenatal indomethacin and ibuprofen and postnatal indomethacin and ibuprofen had been received ($P < 0.0001$), and this persisted at 6 mo of age for those exposed to antenatal indomethacin and postnatal indomethacin and ibuprofen (Fig. 2). Chi square test was used for all analyses.

Light microscopy findings. Glomerular appearance on light microscopy was normal in all groups at 30 days and 6 mo of age. There was no difference in the degree of proximal tubular lumen differentiation at 30 days and at 6 mo of age in any group. Proximal tubular mitoses were more common in control rats at 30 days than treated animals ($P = 0.01$), but this difference was no longer present at 6 mo of age. There was no difference in the extent of proximal tubular epithelial cell drop out in any treated group at 30 days or 6 mo of age. There was no difference in medullary calcifications, medullary inflammation, medullary edema, or papillary edema in the treated groups at 30 days or 6 mo of age.

Glomerular number. There was no significant difference between gender of animals and number of glomeruli at 30 days or 6 mo of age in either control groups ($P = 0.86$ and 0.16 , respectively) or those receiving indomethacin ($P = 0.06$ and 0.57 , respectively) or ibuprofen ($P = 0.33$ and 0.77 , respectively). There was no significant difference between total glomerular volume at 30 days and 6 mo between any of the groups ($P = 0.4$ and 0.5 , respectively) and no significant

Fig. 1. Electron microscopy of glomeruli from control animal (left) and postnatal indomethacin (right) at 6 mo (adult equivalent) showing normal appearance (red arrows) and foot process effacement (green arrows).

Fig. 2. Electron microscopy of glomeruli from control animal (left and top middle) and postnatal indomethacin (right and bottom middle) at 6 mo (adult equivalent) showing humps/bumps (red arrows), splits (green arrows).

difference between total corpuscle volume at 30 days and 6 mo ($P = 0.6$ and 0.8 , respectively). There was no significant difference in the number of glomeruli at 30 days between those rats receiving postnatal indomethacin or ibuprofen ($P = 0.5$). There were no differences in glomerular number between controls and those receiving indomethacin postnatally in each individual group. However, when using a mixed generalized linear model using a Tweedie model type with log link and the Chi square statistics based on a likelihood ratio method, at 6 mo of age those rats having received postnatal indomethacin had significantly less glomeruli compared with all other groups ($P = 0.003$; Table 4). Response variables used were total number of glomeruli at 30 days and at 6 mo. Predictors included the experimental groups examined by main effects

and interaction effects with confounding factors sex, weight (g) at day 1, and weight of kidney (g) at death.

DISCUSSION

This study shows that postnatal administration of indomethacin during the period of ongoing glomerulogenesis in the neonatal rat results in decreased glomerular number in adulthood. The indomethacin-treated rats contain up to 12% fewer nephrons than rats in other groups. Any differences in body and kidney weights at 30 days were no longer evident at 6 mo. The stage of glomerular development in this neonatal rat model is consistent with that of a premature neonate at 24–26 wk gestation, at which time indomethacin or ibuprofen would be given to treat a PDA. With reduced glomerular number as an adult, there is increased risk for hypertension, cardiovascular and renal disease (5, 7, 10, 28–30, 37, 49, 61).

The difference in glomerular number at 6 mo compared with 30 days is consistent with damaged, dysfunctional glomeruli resulting in glomerular loss in adulthood. The EM findings showing smaller glomeruli at 30 days and ongoing foot process effacement both at 30 days and 6 mo support this model. Urinary podocytes have been found in preterm neonates receiving indomethacin supporting the EM findings at 14, 30 days, and 6 mo, of significant glomerular injury (31, 32).

Prematurity itself may be implicated in reduced glomerular number. Histomorphological studies of postmortem renal tissue have shown accelerated postnatal maturation, reduced nephrogenic zone width, reduced renal vesicle formation, and increased glomerular generations compared with age-matched controls (52). Mice delivered 1–2 days preterm have a significant reduction in nephron number (51). Reduced nephrogenic zone width has also been seen in the premature baboon model treated with ibuprofen (49). Rodriguez et al. (48) reported a reduction in the number of glomerular generations at the completion of nephrogenesis, suggesting a nephron deficit.

Table 4. Glomerular number at 6 mo of age

Group	Estimates		95% Wald Confidence Interval	
	Mean	SE	Lower	Upper
Group 6 (Postnatal ibuprofen)	34066.38	1861.447	30418.01	37714.75
Group 7 (Postnatal ibuprofen)	35052.99	1731.171	31659.95	38446.02
Group 4 (Postnatal indomethacin)	29735.73	1476.050	26842.73	32628.74
Group 5 (Postnatal indomethacin)	29132.44	1470.396	26250.52	32014.36
Group 3 (Antenatal ibuprofen)	31317.63	1656.669	28070.62	34564.64
Group 2 (Antenatal indomethacin)	32222.42	1568.270	29148.67	35296.17
Control (Group 1)	33760.18	1750.010	30330.22	37190.13

Covariates appearing in the model are fixed at the following values: body weight day1 = 7.06 g; left kidney weight 6 mo = 2.0537 g. An interaction between drug and timing of the administration was found ($P = 0.003$).

Along with the changes in nephrogenic zone width and glomerular generations, up to 13% of glomeruli in the preterm kidney were noted to have enlarged Bowman's space and shrunken glomerular tufts (52). With reduced capillarization and impaired vascular development/injury, these glomeruli may be nonfunctional (24, 25).

Indomethacin may often be prescribed at the same time as ampicillin and gentamicin in the first few days of life, which have both been shown to affect nephron development and function (13, 17–20, 35, 38, 40, 43, 57). Minimizing the coadministration of nephrotoxic medications during this active period of nephrogenesis in the preterm infant would now appear to be an important consideration in neonatal intensive care. Alternative options to indomethacin and ibuprofen should also be considered. Paracetamol has been reported in a number of case series and small randomized controlled trials to be as effective as oral ibuprofen to close a patent ductus arteriosus (14, 39, 44, 55). Paracetamol works as an inhibitor of the peroxidase component of the prostaglandin-H2 synthetase pathway, whereas indomethacin and ibuprofen work on the cyclooxygenase pathway.

The ductus arteriosus is kept patent in the fetus due to low partial pressure of oxygen and circulating or locally produced prostaglandins and nitric oxide. Inhibition of prostaglandin synthesis will result in closure of the duct and this is brought about by inhibition of cyclooxygenase (22). Inhibition of cyclooxygenase by selective COX-1 and COX-2 inhibitors, including indomethacin, has been shown in rats, mice, and lambs to constrict the patent ductus arteriosus (9, 12, 50, 56). This study used slightly higher doses of both indomethacin and ibuprofen compared with that in the clinical setting. Usual doses of indomethacin would be 0.2 mg/kg on the first day followed by 0.1 mg/kg daily for 5 days or 0.2 mg/kg 12 hourly for 3 doses and for ibuprofen 10 mg/kg for one dose followed by 5 mg/kg for 2 daily doses or 20 mg/kg for one dose followed by 10 mg/kg for 2 daily doses. However, the doses used in this animal study are relatively comparable and neonates may receive two courses of either medication to try and successfully close a PDA, and as such the doses used in this animal study are similar to the additive nephrotoxic exposure to these medications neonates would receive.

In conclusion, early postnatal administration of indomethacin results in a reduced glomerular number in the adult rat. This effect was not seen with ibuprofen. This study will not be replicable in the human preterm neonate, and as such the use of indomethacin for a PDA should be used judiciously, and perhaps ibuprofen (oral or intravenously) should be considered the first line treatment for a PDA until potential other treatments, such as paracetamol, become available.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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