CALL FOR PAPERS | Programming Normal Renal Development and Modeling Disease Pathogenesis

Effect of intra-amniotic lipopolysaccharide on nephron number in preterm fetal sheep

Robert Galinsky,1 Timothy J. M. Moss,1,3 Lina Gubhaju,2 Stuart B. Hooper,1 M. Jane Black,2,* and Graeme R. Polglase1,3*

1The Ritchie Centre, Monash Institute of Medical Research, 2Department of Anatomy and Developmental Biology, and 3Department of Obstetrics and Gynecology, Monash University, Clayton, Victoria, Australia

Submitted 28 January 2011; accepted in final form 18 May 2011

Galinsky R, Moss TJ, Gubhaju L, Hooper SB, Black MJ, Polglase GR. Effect of intra-amniotic lipopolysaccharide on nephron number in preterm fetal sheep. Am J Physiol Renal Physiol 301: F280–F285, 2011. First published May 18, 2011; doi:10.1152/ajprenal.00066.2011.—Chorioamnionitis is an antecedent of preterm birth. We aimed to determine the effect of experimental chorioamnionitis in fetal sheep during late gestation on 1) nephron number, 2) renal corpuscle volume, and 3) renal inflammation. We hypothesized that exposure to chorioamnionitis would lead to inflammation in fetal kidneys and adversely impact on the development of nephrons, leading to a reduction in nephron number. At ∼121 days of gestation (term ∼147 days), pregnant ewes bearing twin or singleton fetuses received a single intra-amniotic injection of lipopolysaccharide (n = 6; 3 singletons, 3 twins); controls were either untreated or received an intra-amniotic injection of saline (n = 8; 4 singletons, 4 twins). One twin was used from each twin-bearing ewe. At ∼128 days of gestation, fetuses were delivered via Caesarean section. Kidneys were collected and stereologically analyzed to determine nephron number and renal corpuscle volume. Renal inflammation was assessed using immunohistochemistry. Experimental chorioamnionitis did not affect body weight or relative kidney weight. There was a significant reduction in nephron number but no change in renal corpuscle volume in LPS-exposed fetuses relative to controls. Overall, nephron number was significantly reduced by 23 and 18% in singleton and twin LPS-exposed fetuses relative to controls. On average, nephron number was not different between groups. Importantly, this study demonstrates that exposure to experimental chorioamnionitis adversely impacts on nephron number in the developing fetus.

intrauterine inflammation; chorioamnionitis; renal development; fetal inflammatory response; preterm birth

CHORIOAMNIONITIS IS A COMMON antecedent of preterm birth (15) in humans; it is frequently present when preterm birth occurs before 30 wk of gestation (32). Chorioamnionitis may manifest as either a clinical or subclinical condition, the latter being the most common, whereby the chorion, amnion, and placenta become inflamed, often as a result of bacterial infection (49). This can give rise to the fetal inflammatory response syndrome (FIRS), characterized by funisitis, fetal vasculitis, and an increase in of proinflammatory cytokines in fetal blood and amniotic fluid (16, 48, 49).

Clinical and experimental studies demonstrated that FIRS affects the neonatal lungs, brain, and thymus (31, 36, 38, 40, 44, 52, 55). In humans, there is some evidence that chorioamnionitis is associated with oligohydramnios, suggestive of a redistribution of blood flow away from the kidneys as a result of FIRS (56). In addition, chorioamnionitis was independently associated with renal and electrolyte abnormalities in a cohort of preterm neonates treated with indomethacin (23), suggesting chorioamnionitis may have adverse effects on renal development. However, there are no experimental data about the effects of chorioamnionitis or FIRS on renal development.

Sheep are a common species used to study the effects of intrauterine perturbations on kidney development (17, 34, 57). The timing of nephrogenesis in fetal sheep closely resembles that in humans, whereby the majority of nephrons are formed during the final third of gestation, with no new nephrons formed after term birth (14). We propose that newly formed and immature glomeruli in the fetal kidney during the late stages of nephrogenesis would be vulnerable to intrauterine inflammation. We hypothesized that experimental chorioamnionitis in sheep, induced by an intra-amniotic injection of lipopolysaccharide (LPS) (24), would induce renal inflammation and lead to a reduction in nephron number. The aim of the current study was to determine the effect of experimental chorioamnionitis in fetal sheep during late gestation on 1) nephron number, 2) renal corpuscle volume, and 3) renal inflammation. We studied singleton and twin fetuses because twins have been shown to contain fewer glomeruli (34), potentially making them more vulnerable to any insult that affects the kidney.

MATERIALS AND METHODS

Induction of experimental chorioamnionitis. All procedures were approved by the Monash University Animal Ethics Committee. At 121 ± 1 (means ± SD) days of gestation (days: term ∼147 days), pregnant ewes bearing singleton or twin fetuses received an intra-amniotic injection of LPS (Escherichia coli 055:B5, 10 mg; Sigma, NSW, Australia; n = 6; 3 singletons, 3 twins) as previously described (24). The single intra-amniotic 10-mg dose was chosen because it has previously been shown to induce inflammation and injury in many organs of fetal sheep (4, 10, 26, 30, 31). Control ewes (n = 8; 4 singletons, 4 twins) received either intra-amniotic saline (n = 6) or no
intervention (n = 2). Previous studies showed that a single intra-amniotic saline injection has no effect on fetal well-being compared with no intervention (39). Hence, after confirming that there was no difference in nephron number, renal corpuscle volume, and glomerular density in the control fetuses that did not receive intra-amniotic saline relative to those that did, we pooled the data from both cohorts to act as our controls. In the control group, one twin was examined from each twin-bearing ewe.

At 128 ± 1 days, ewes were anesthetized, fetuses were delivered by Caesarean section, and a fetal cord blood sample was collected via the umbilical vein before the fetus was euthanized using an overdose of pentobarbitone (100 mg/kg iv, 153 Valabarb; Jurox, Rutherford, Australia). Intrauterine inflammation was confirmed by the presence of edematous fetal membranes and/or funisitis, characteristic of this model (44).

**Kidney sampling, processing, embedding, and sectioning.** The fetal kidneys were excised and weighed. The right kidney was cut through the hilus in both vertical and horizontal planes, giving four approximately equal quarters, and immersion fixed using 10% buffered formalin. One kidney quarter and its diagonally opposite counterpart were randomly selected and sliced into 2-mm-thick slices; every third slice was sampled, processed, and embedded in glycolmethacrylate (GMA) resin (Technovit 7100 resin; Heraeus Kulzer, Hanau, Germany). The GMA blocks were exhaustively sectioned at 20 μm using a Leica RM 2165 microtome (Leica Microsystems, Nussloch, Germany) fitted with a glass-cutting blade. Beginning with a random number (between 1 and 10), every 10th and 11th section was collected and stained with hematoxylin and eosin.

**Stereological estimation of nephron number and mean renal corpuscle volume.** Nephron number and renal corpuscle volume, a surrogate for glomerular tuft volume, were determined using unbiased stereological methods (41). Since the kidneys were immersion fixed, we chose to measure renal corpuscle volume rather than glomerular tuft volume as the latter is affected by lack of perfusion. An unbiased physical disector/fractionator technique (described by Bertram (3) and Mitchell et al. (34)), using the 10th and 11th kidney sections, was used to determine glomerular number and thereby nephron number. Glomeruli depicting a defined Bowman’s space and capsule were included to determine glomerular number and thereby nephron number. Glomeruli depicting comma and S-shaped bodies were not counted.

The mean renal corpuscle volume (inclusive of the glomerular tuft and Bowman’s space and capsule) was obtained by dividing the volume density of the renal corpuscle by the numerical density of the renal corpuscle (34).

**Assessment of inflammation within the renal corpuscle.** The presence of inflammatory cells within the renal corpuscle was assessed by immunohistochemically localizing the common leukocyte antigen, CD45. Paraffin-embedded 5-μm sections were deparaffinized, rehydrated, and washed in phosphate-buffered saline (PBS) solution. Sections were immersed in 0.01 M sodium citrate (pH 6) and heated in a microwave for 20 min (2 × 10 min) to facilitate antigen retrieval. Unmasking of antigenic sites was performed using 3% H2O2 before nonspecific binding was blocked by incubation in a 0.05 M Tris-HCl solution with 10% normal horse serum and 0.1% bovine serum albumin for 30 min. The sections were incubated with CD45 mouse anti-sheep primary antibody (AbD serotec, Queensland, Australia; 1:50 dilution) overnight at room temperature. Sections were washed in PBS solution containing 0.1% Tween 20 and incubated with goat anti-mouse immunoglobulin G secondary antibody (Dako, Botany, NSW; 1:500 dilution) for 1 h. The primary antibody was omitted from negative control sections. Sections were then washed in PBS solution containing 0.1% Tween 20 and incubated with horseradish peroxidase solution (1:200 dilution) for 30 min. Sections were washed in PBS solution and incubated in 3,3 diaminobenzidine tetrahydrochloride to detect antibody binding. All sections were counterstained with hematoxylin.

To quantitate CD45-positive immunostaining, the number of positively stained cells in a minimum of 20 renal corpuscles per animal was counted and averaged based on the technique outlined by Huang et al. (21). Renal corpuscles were randomly selected based on the method outlined by Nyengaard and Marcussen (42). Data are expressed as the mean number of positive cells/number of renal corpuscles analyzed per kidney (21).

**Statistical analyses.** Data are presented as means ± SE. Statistical analyses were undertaken using Graphpad Prism software (v.5.00; GraphPad Software, San Diego, CA). Data from singleton and twin fetuses were analyzed separately because twins have fewer glomeruli, potentially making them more susceptible to an insult that affects the kidney (34). Group data were compared by two-way ANOVA with LPS vs. control and singleton vs. twin as separate factors. The Bonferroni post hoc test was used to compare groups where an interaction was identified by two-way ANOVA.

In agreement with previously published studies (7, 20, 22, 33), we observed no effect of sex on nephron number and renal corpuscle volume (P = 0.48 and 0.48, respectively; 2-way ANOVA). In addition, the effect of LPS exposure on nephron number and renal corpuscle volume did not differ in males relative to females for nephron number (P = 0.48) or renal corpuscle volume (P = 0.55); hence, data from males and females were pooled in the stereological analyses. For CD45 quantification, control and LPS groups were compared using an unpaired Student’s t-test. Statistical significance was accepted for P < 0.05.

**RESULTS**

**Fetal blood gases, body and kidney weight.** Fetal umbilical cord venous blood gas parameters (pH, Pco2, Po2, So2) were all within the normal range for fetal sheep and were not different between LPS-exposed fetuses compared with controls (Table 1). Body weights were not different between control and LPS groups; however, twins were lighter than singletons (P < 0.01; Table 2). Absolute and relative kidney weights were not different between the LPS and control groups, but relative kidney weight was higher in twins compared with singletons (P < 0.01; Table 2).

**Nephron number and glomerular density.** Total nephron number in singletons and twins was lower in LPS-exposed fetuses, by 23 and 18%, respectively, compared with controls (P < 0.05; Fig. 1A). There was no difference in nephron number (P > 0.05; Table 1).

**Table 1. Umbilical vein blood gas parameters in twin and singleton, control and LPS lambs**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Twin</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Singleton</td>
<td>Twin</td>
<td>LPS vs. Control</td>
</tr>
<tr>
<td>pH</td>
<td>7.12 ± 0.03</td>
<td>7.20 ± 0.02</td>
<td>0.13</td>
</tr>
<tr>
<td>Pco2 (mmHg)</td>
<td>75.0 ± 4.5</td>
<td>69.1 ± 6.4</td>
<td>0.35</td>
</tr>
<tr>
<td>Po2 (mmHg)</td>
<td>20.3 ± 4.3</td>
<td>21.4 ± 4.0</td>
<td>0.72</td>
</tr>
<tr>
<td>So2 (%)</td>
<td>34.7 ± 10.5</td>
<td>50.7 ± 11.0</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Data are means ± SE. Umbilical vein blood gas parameters in twin and singleton, control and LPS lambs at 128 ± 1 days (term ~147 days).
number between twin and singleton fetuses and the effect of LPS treatment on nephron number was not different between twins and singletons.

Glomerular density (the number of glomeruli per unit kidney volume) was significantly reduced in LPS-exposed fetuses compared with controls in both singletons and twins (P < 0.03; Fig. 1B). Twin fetuses had a greater glomerular density compared with singletons (P < 0.01; Fig. 1B). Glomerular number relative to body weight was reduced in LPS-exposed fetuses compared with controls in both singletons and twins (P < 0.03; Fig. 1C). Twin fetuses had more glomeruli relative to body weight than singletons (P < 0.04; Fig. 1C).

Renal corpuscle volume. Renal corpuscle size tended to be greater in the LPS-exposed singletons compared with control singletons, but this failed to reach significance (P = 0.06; Fig. 2). There was a reduction in renal corpuscle volume in twins compared with singletons, attributable to a reduction in renal corpuscle volume in the LPS-exposed twin kidneys relative to LPS-exposed singletons (P < 0.01; Fig. 2).

Common leukocyte antigen (CD 45) immunohistochemistry. The average number of CD45-positive cells per renal corpuscle was not different between the LPS-exposed and control groups (3.1 ± 0.6 and 3.8 ± 0.4 positively stained cells, respectively; P = 0.31).

**DISCUSSION**

Experimental chorioamnionitis, induced by intra-amniotic LPS administration, resulted in a significant reduction in nephron number in singleton and twin fetal sheep. The structural alterations to kidney development that we observed are in accordance with altered structural development of other fetal organ systems using this model (4, 10, 25, 28, 31, 36, 54).

In humans, the process of nephrogenesis commences early in gestation (week 9) and is complete by ~34 to 36 wk of gestation, with the majority of nephrons formed in the third trimester of pregnancy (8, 18, 50). Sheep are a good model of human nephrogenesis as nephrogenesis occurs between 30 and 100 days of gestation, with the majority of nephrons formed within the third trimester of pregnancy (28, 31, 36, 54).

| Table 2. Body weight, absolute and relative kidney weight in twin and singleton, control and LPS lambs |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                | Control                        | LPS                            | P values                        |
|                                | Singleton                      | Twin                           | LPS vs. Control                | Singleton vs. Twin             | Interaction |
| Male:female                    | 2:2                            | 2:2                            | 1:2                            | 1:2                            | 0.22        | <0.01 | 0.12 |
| Body wt, kg                    | 3.45 ± 0.13                    | 2.74 ± 0.12                    | 3.94 ± 0.26                    | 2.68 ± 0.12                    | 0.92        | 0.16  | 0.33 |
| Kidney wt, g                   | 10.69 ± 1.20                   | 11.18 ± 0.61                   | 9.77 ± 0.72                    | 12.30 ± 1.25                   | 0.88        | <0.01 | 0.07 |
| Relative kidney wt, g/kg       | 3.08 ± 0.29                    | 4.09 ± 0.20                    | 2.51 ± 0.29                    | 4.58 ± 0.28                    | 0.22        | <0.01 | 0.12 |

Data are means ± SE. Body weight, absolute and relative kidney weight in twin and singleton, control and LPS lambs at 128 ± 1 days (term ~147 days).
130 days with the majority of nephron formation occurring within a 40-day period (80–120 days) (14, 46, 53). Nephrogenesis initially involves branching of the ureteric tree (with nephrons formed at the branch points) followed by glomerular arcade formation at terminal branch points (43). We induced experimental chorioamnionitis during late gestation (121 days), a time at which we expect branching morphogenesis would have ceased but nephrons would be continuing to form on the terminal branch tips. Hence, the findings of the present study suggest that experimental chorioamnionitis is adversely impacting on the formation of nephrons at this stage of glomerular arcade formation. How this occurs is unknown, although altered hemodynamics and/or local inflammation within the kidney are potential mediators. Certainly, the reduction in nephron number is not due to overall growth restriction or impaired fetal wellbeing because body size and blood gas status at the time of delivery were not affected by LPS exposure, consistent with previous observations using this model (37, 39).

In a study of women with preterm premature rupture of membranes, oligohydramnios was associated with signs of intrauterine inflammation (56). This suggests a reduction in fetal renal function that may be secondary to a redistribution of blood flow away from the fetal kidneys, because fetal urine production is a major contributor to amniotic fluid volume (35). Similar observations have been made in adults with sepsis, where oliguria is thought to be a manifestation of renal hypoperfusion (45). Therefore, it is conceivable that nephrogenesis may have been inhibited in our LPS-exposed fetuses as a result of renal hypoperfusion.

Alternatively, the reduction in nephron number may have resulted from renal inflammation. Although we did not observe increased white blood cells in the kidneys of fetuses 7 days after LPS treatment, we cannot be certain that renal inflammation did not occur in the immediate period following the LPS administration. Indeed, fetal lung cytokine mRNA levels are increased at 15 h after a single intra-amniotic injection of LPS, they peak at 2 days after injection (27, 28), but are similar to control values 7 days after intra-amniotic LPS exposure (26, 30). In future studies, it would be interesting to assess renal hemodynamics in fetuses exposed to LPS and determine whether there is renal inflammation [accompanying the systemic inflammation (29)] in the first few days after intra-amniotic LPS administration.

There has been a number of studies demonstrating differential programming to in utero insults between sexes (13), and this has been observed in the developing sheep kidney as a result of maternal nutrient restriction (11). However, in the present study, the response of the kidney to inflammation in utero (in regards to nephron number and renal corpuscle size) did not differ between male and female fetal sheep. When analyzed using a two-way ANOVA, there was no difference in the response to LPS exposure between the sexes (nephron number: \( P_{LPS \times sex} = 0.48 \) and renal corpuscle volume: \( P_{LPS \times sex} = 0.55 \)).

The findings of this study may have important implications to the short- and long-term renal health of individuals born preterm. Chorioamnionitis is a common antecedent of preterm births, especially those before 30 wk gestation (32). The immature kidneys of the preterm infant (especially those born extremely preterm) are particularly vulnerable to renal dys-

function (1, 2, 6, 9). Recent studies show that nephrogenesis continues after preterm birth but of concern, glomerular abnormalities are often present (19, 47, 51). The findings of this study demonstrate that the adverse effects of preterm birth on nephrogenesis may be compounded when the infant has been exposed to chorioamnionitis in utero. Nephron number is already compromised at birth and the kidneys then have the added stress of nephrogenesis occurring in the extrauterine environment, where they are exposed to a number of potential nephro toxic factors such as altered hemodynamics, hypoxia, and exposure to nephro toxic medications. Importantly, in this regard, a multiple logistic regression analysis of 2,508 preterm neonates, treated with indomethacin, showed a significant correlation between intrauterine inflammation and prevalence of renal and electrolyte abnormalities (23), thus suggesting that intrauterine inflammation in concert with postnatal indomethacin treatment can lead to renal dysfunction. Certainly our results support the idea that prematurity, when complicated with chorioamnionitis, is likely to exacerbate postnatal renal dysfunction.

In the current study, twinning resulted in a significant reduction in body weight at the time of delivery (128 ± 1 days); however, twins had a significantly increased kidney weight relative to body weight and no change in nephron number, suggesting that growth of the kidney was not affected by twinning. While these observations are supported by previous studies (5, 12), they contrast with previous studies from our laboratory where a significant reduction in body weight, kidney weight, and nephron number has been observed in twin controls relative to singleton controls at 140 days gestation (34). Why nephron number was not significantly affected in the normally grown twin fetuses relative to singletons in the present study compared with our previous study may relate to the gestational time point when nephron number was examined and/or the comparative increase in kidney growth in twins at the time point examined. This may also account for differences relating to the size of the renal corpuscle between the two studies (34).

In conclusion, this study demonstrates that exposure to experimental chorioamnionitis adversely impacts on nephron number, which may in turn have long-term deleterious effects on renal health. These findings highlight the need for further research into the effect of intrauterine inflammation on 1) the developing kidney, 2) postnatal renal function, and 3) the potential risk of increased blood pressure and renal dysfunction later in life.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance of Histological Services at Monash University and Monash Institute of Medical Research.

GRANTS

This study was funded by a National Heart Foundation of Australia (NHFA) grant (G. R. Polglase), Australian Postgraduate Award (R. Galinsky), National Health and Medical Research Council of Australia (NHMRC) Career Development Award (T. J. M. Moss, 303261), NHFA/NHMRC Fellowship (G. R. Polglase), and an NHMRC Fellowship (S. B. Hooper).

DISCLOSURES

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