Accelerated senescence in kidneys of low-birth-weight rats after catch-up growth

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Accelerated senescence in kidneys of low-birth-weight rats after catch-up growth. Am J Physiol Renal Physiol 297: F1697–F1705, 2009. First published October 14, 2009; doi:10.1152/ajprenal.00462.2009.—Epidemiological studies show a strong association between low birth weight and hypertension, renal, and cardiovascular disease, especially after catch-up growth. Senescence is an important contributor to the progression of chronic disease. Developmentally programmed premature senescence may be a link among low birth weight, catch-up growth, and adult disease. Low birth weight was induced by feeding pregnant rats a low-protein diet from day 12 of gestation to 10 days postdelivery. Low- and normal-birth-weight male offspring were weaned onto regular or high-calorie diets to enhance catch-up growth. Kidneys and hearts of offspring were analyzed for RNA and protein markers of stress-induced senescence (p16, p21, p53, Rb). Markers of mitochondrial stress (p66Shc) and activation of endoplasmic reticulum protein secretion (Ero1α) were analyzed as regulators of reactive oxygen species generation. Reactive oxygen species are known to be associated with premature aging. Senescence markers were not different in low- or normal-birth-weight kidneys at birth. During rapid catch-up growth, p16 and p21 increased significantly in low-birth-weight kidneys and hearts (P < 0.01). Renal p16 levels increased progressively and were significantly higher in low-birth-weight kidneys at 3 and 6 mo (P ≤ 0.02). Renal p66Shc and Ero1α were significantly higher in low- compared with normal-birth-weight kidneys at 6 mo, suggesting reactive oxygen species generation (P ≤ 0.03). Low-birth-weight rats exhibit accelerated senescence in kidneys and hearts after rapid catch-up growth, a likely important link between early growth and subsequent hypertension, renal, and cardiovascular disease.

cardiovascular disease; kidney disease; hypertension

Epidemiological studies show a convincing association of low birth weight (LBW) with hypertension, diabetes, obesity, renal, and cardiovascular diseases, especially after early “catch-up” growth (16, 18, 28, 58). The frequent coexistence of these disorders, which are also associated with aging, may reflect a common underlying mechanism. One such mechanism is the far-reaching impact of the fetal environment and early postnatal development on programming of adult disease, potentially through premature aging.

In humans, evidence of senescence, as measured by reduced telomere length or increased p16 expression in vascular cells, circulating leukocytes, and aging or injured kidneys, is consistent with a role for senescence in the pathophysiology of atherosclerosis, heart failure, and renal allograft dysfunction (13, 19, 22, 36, 42, 43). The kidney is one of the organs that ages fastest, and expression of the senescence marker p16 has been shown to correlate best with renal aging but has not been studied in association with birth weight (37). With regard to birth weight, lymphocyte telomere lengths were not different in LBW and normal-birth-weight (NBW) British children at birth, but they were significantly shorter in LBW compared with NBW Bangladeshi children at age 5 yr, suggesting telomeres may shorten faster in LBW children after birth (1, 50). Similarly, telomere length was similar in LBW and NBW rats at weaning, but in LBW adult male rats, catch-up growth was associated with shorter life span and shorter renal telomeres compared with slow postnatal growth (23, 33). Calorie restriction in normal mice is associated with slower aging, reduced p16 expression, and fewer pathological changes in the kidney and heart (24). Prenatal malnutrition followed by catch-up growth may therefore accelerate senescence over time, whereas postnatal calorie restriction retards senescence.

Cellular senescence is a stress response which results in permanent withdrawal from the cell cycle (12). Senescence may result from two separate processes which converge on a common pathway of growth arrest, i.e., cell replication (relicative senescence) associated with telomere shortening, or DNA damage (extrinsic, stress-induced senescence), largely mediated by reactive oxygen species (ROS) and activation of the p16INK4a and ARF pathways (61). Telomere shortening is therefore not essential to stress-induced premature senescence (6, 35). DNA damage from various insults activates the p53-p21 and p16-pRb pathways (6, 61). The p53-p21 pathway induces apoptosis, but when coordinated with the p16 pathway, p21 and p16 maintain hypophosphorylation of Rb and induce senescence (7). The free radical theory of aging states that aging is associated with accumulation of ROS (5, 27). A critical element along this pathway is p66Shc, which regulates mitochondrial ROS production (5, 27). Intriguingly, reduction in p66Shc expression is associated with 30% increased longevity in mice and resistance to oxidative injury (9, 39). More recently, the regulation of protein secretion at the level of the endoplasmic reticulum (ER) has also been tied to aging because of decreased efficiency of ER protein folding in older animals, and increased production of ROS by the ER protein folding machinery itself, dependent on the ER oxidoreductase Ero1α (20, 41).

To investigate the potential role of premature aging in programming of renal and cardiovascular disease, we analyzed expression of senescence markers p16, p21, p53, and Rb, mitochondrial p66Shc, and ER oxidoreductase Ero1α in kidneys and hearts of LBW and NBW rats at birth, during and after catch-up growth, on normal or high-calorie diets.

Methods

Animals. This study was approved by the Health Sciences Animal Care and Use Committee at the University of Alberta. Timed pregnant female Sprague-Dawley rats were obtained on days 9–10 of gestation

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(Harlan, Madison, WI) and were housed individually with free access to chow and tap water. After acclimatization, pregnant dams were placed on an isocaloric low-protein (5%) or normal-protein (19%) diets from day 12 of gestation (nos. 5767 and 5755, Purina TestDiets, Gray Summit, MO) to induce LBW or NBW in offspring. This protocol is known to result in reduced nephron number by 25–30%, adult hypertension, and reduced longevity in LBW offspring (55). Kidney sections of a LBW and NBW animal are shown in Supplementary Fig. 1 (all supplementary material for this article is available on the journal web site). Gestational diets were continued until postnatal day 10, i.e., the end of nephrogenesis. Lactating females were switched to normal chow on postnatal day 10. NBW pups were weaned at 3 wk and LBW pups at 4 wk because they were smaller. LBW and NBW males were randomly selected from 8 and 11 litters to minimize any litter effect and divided into two groups receiving either a regular diet (R; 19% protein, 22% fat) or a high-calorie/high-fat diet (H; 15% protein, 40% fat; nos. 5755 and 5342, Purina TestDiets), resulting in the following groups: NR = NBW, regular diet; NH = NBW, high-calorie/high-fat diet; LR = LBW, regular diet; LH = LBW, high-calorie/high-fat diet. Weaned animals were housed in pairs with free access to chow and tap water. At least four animals were analyzed per group with the exception of two in the LR 6-mo group. Gene expression and tissue analysis were restricted to males because the programmed phenotype is known to be more severe in males (23, 60). Rats were weighed at birth as whole litters and singly thereafter. From weaning onward, weights were tracked individually. Newborn pups were euthanized by decapitation. From weaning onward, animals were euthanized by intraperitoneal injection of an excess of pentobarbital sodium (CEVA Sante Animale, La Ballastiere, France). At the time of harvesting, organs were snap frozen in liquid nitrogen for RNA and protein extraction or in Tissue-Tek OCT compound (Sakura Finetek, Torrence, CA) for immunofluorescence. Real-time RT-PCR. Total RNA was extracted from tissues using a RNeasy Mini Kit (Qiagen, Mississauga, ON) for kidneys and RNeasy Fibrous Tissue Mini Kit (Qiagen) for hearts. Two micrograms of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Burlington, ON). Expression of senescence markers p16, p21, and p53 and the mitochondrial stress marker p66Shc were analyzed by real-time RT-PCR using 20 ng of cDNA for each sample/gene and 100 ng for p16. Oligonucleotide primers and probes were purchased as fluorogenic primer/probe sets (Applied Biosystems, Foster City, CA) with the exception of the housekeeping gene HPRT, which was purchased as custom oligonucleotides (Sigma-Genosys, Oakville, ON). Primers are shown in Supplementary Table 1. Simplex reactions were performed in triplicate for the gene of interest and for the endogenous housekeeping gene (HPRT-FAM) in 96-well MicroAmp plates using the 7900HT fast real-time PCR System (Applied Biosystems). Gene transcript levels were calculated according to the \( \Delta \Delta C_T \) method and expressed as percent HPRT (29). If CT was not reached by 40 cycles of amplification, expression was considered below the limit of detection.

Protein analysis. Nuclear and cytoplasmic extracts were obtained from whole kidneys of 6-mo-old rats using an NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL). Twenty to fifty micrograms of protein extract were separated by 8–12% SDS-PAGE and transferred overnight to polyvinylidene difluoride membranes (GE Healthcare). Antibodies against p16 (sc-1207), Rb (sc-50), and actin was used as a loading control (31). Densitometry was performed using Quantity One software (Bio-Rad, Mississauga, ON). Protein expression is shown graphically as results for rats in each birth weight/diet group to demonstrate increased variability of expression among LBW rats. Each representative blot shows two animals from each group. Statistical analysis was performed with all LBW compared with all NBW kidneys combined.

Immunofluorescence for p16 was performed on 4-μm cryosections of two rat kidneys from each birth weight/diet group and detected using Alexa Fluor 488 goat anti-rabbit (Invitrogen, Carlsbad, CA) (30).

Statistical analysis. Rat weights and gene expression were analyzed using Student’s t-test and ANOVA assuming significance at \( P \leq 0.05 \) (23a). At the 6-mo time point, because of numbers, rats were analyzed by birth weight category (\( n = 6 \) in the LBW group, i.e., 2 LR + 4 LH, and \( n = 8 \) in the NBW group, i.e., 4 NR and 4 NH diets), pooling rats from both diet groups within each birth weight group.

## Results

**LBW rats experience early catch-up growth.** Mean birth weights were 4.9 ± 0.17 g in LBW and 6.1 ± 0.08 g in NBW litters. Mean weights on day 10 were 8.0 ± 2.53 g in LBW and 23.9 ± 2.15 g in NBW litters (\( P < 0.001 \)). LBW pups gained weight rapidly upon reintroduction of a normal maternal diet at day 10 but did not become obese (Fig. 1). The rate of change in weight in LBW rats increased rapidly between day 10 and week 6, indicating the period of catch-up growth, after which growth trajectories remain relatively parallel. Mean percent change in weight from day 10 to week 6 was 689 ± 82% in NBW and 1465 ± 235% in LBW rats (Fig. 1A, inset) (\( P < 0.0001 \)). From Fig. 1A it can be seen that there are two “windows” within this period of catch-up growth in the LBW rats, one from day 10 to weaning and a second from weaning to week 6. Analysis of rate of growth in the first 2 wk after weaning revealed a greater percent weight gain in NBW compared with LBW rats (68.1 ± 2.1% vs. 60.1 ± 4.7%, \( P < 0.001 \)). This indicates that the fastest period of catch-up growth in LBW rats between day 10 and week 6 was the early period immediately after switch of maternal diet at day 10. Among NBW rats, weight gain increased steadily from birth to weaning and accelerated after weaning. Rats in all groups experienced rapid growth after weaning. At 6 mo, weight of LBW rats on a regular diet (LR) was similar that of NBW rats on a regular diet (LR) was similar that of NBW rats on a regular diet (NR) or a high-calorie/high-fat diet (NH), although LBW rats on a high-calorie/high-fat diet (LH) remained lighter (Fig. 1B). Within birth weight groups, rats on high-calorie/high-fat (H) diets did not gain more weight than those on regular (R) diets.

**Senescence markers in kidneys and hearts of young rats increase during catch-up growth.** p16, p21, and p53 were analyzed by real-time RT-PCR in hearts and kidneys of young male rats at birth and weaning (Fig. 2). The weaning time point was chosen to minimize confounding by other factors: hypertension and tissue injury have not yet developed in this model (52, 55); catch-up growth is ongoing; future diets have not yet been instituted; therefore, observed changes should reflect the impact of birth weight and catch-up growth. At birth, expression of p16, p21, and p53 was not different between LBW and NBW hearts or kidneys. Transcripts of p16, p21, and p53 all decreased from birth to weaning in NBW hearts and kidneys and in LBW kidneys. In LBW hearts, p16 and p21 expression did not decrease. At weaning, expression of p16 and p21 in both hearts and kidneys was significantly increased in LBW rats (Fig. 2) (\( P < 0.01 \)), suggesting accelerated senescence associated with postnatal catch-up growth. In contrast, p53 expression was not significantly different between groups.
Increasing expression of p16 in LBW kidneys with age. The senescence marker that best correlates with renal aging is p16 (37). Renal p16 increased progressively and was consistently higher in LBW compared with NBW rats from weaning to 3 and 6 mo (Fig. 3) (\(P < 0.02\)) despite higher variability of p16 expression in LBW kidneys. Among NBW, but not LBW rats, p16 expression was significantly higher in those on H compared with R diets (\(P < 0.02\)). In contrast, expression of p53 and p21 was not different between LBW and NBW kidneys at 3 or 6 mo (Supplemental Fig. 2).

Elevated p16 and Rb protein expression in LBW kidneys with age. Expression of p16 protein was significantly higher in nuclear extracts from 6-mo kidneys of LBW compared with NBW rats (Fig. 4) (\(P < 0.03\)). Consistent with this, immunofluorescence demonstrated a qualitative increase in nuclear p16 staining in LBW compared with NBW kidneys at 6 mo (Fig. 5). Similarly, expression of Rb showed a significant increase in hypophosphorylated (active) Rb relative to the phosphorylated (inactive) Rb in LBW kidneys (Fig. 6) (\(P < 0.01\)), consistent with suppression of gene transcription by hypophosphorylated Rb contributing to senescence in LBW kidneys (59).

Increased expression of genes associated with ROS production in LBW rat kidneys. Expression of mitochondrial p66Shc was analyzed in newborn, weanling, and 6-mo kidneys by real-time RT-PCR (Fig. 7). Transcript levels were higher in NBW kidneys at birth (\(P < 0.03\)), but by 6 mo expression was higher in LBW kidneys (\(P < 0.02\)). Similarly, expression of Er1α protein in 6-mo kidney cytoplasmic extracts was increased in LBW kidneys (Fig. 8) (\(P < 0.001\)). Taken together, these findings suggest increased ROS generation on LBW kidneys with time. We examined expression of the antioxidant enzymes MnSOD and GR and did not find any significant change in cytoplasmic extracts although MnSOD tended to be reduced in LBW kidneys (Supplemental Fig. 3).

DISCUSSION

We have shown for the first time that expression of p16, an established biomarker of stress-induced senescence, is increased in kidneys and hearts of LBW rats during and after a period of rapid catch-up growth. These data suggest that senescence is accelerated in LBW animals and continues to increase with age, likely contributing to the phenotype of premature cardiovascular and renal disease and early mortality.
Expression of p16, p21, and p53 were not different between LBW and NBW hearts or kidneys at birth. Premature renal senescence and growth arrest may therefore not be a major contributor to the observed congenital reduction in nephron number seen in this model; however, this deserves further study as nephrogenesis does continue after birth in the rat.

The significant increase in p16 expression in LBW kidneys was apparent from weaning and increased markedly from 3 to 6 mo. It is difficult to dissect the relative contributions of prenatal programming from growth rate and final body size/composition in the LBW rats. The two windows of rapid growth in LBW rats between day 10 to weaning and weaning to 6 wk suggest two discrete periods of catch-up growth. Rapid growth also occurred in the NBW rats between weaning and 6 wk, however, suggesting that the major difference in catch-up between LBW and NBW rats occurred between day 10 and weaning, i.e., soon after restoration of a normal maternal diet. This early rapid catch-up period is likely to have been the critical window leading to acceleration of senescence in LBW rats. Subsequent growth rates were more similar in NBW and LBW groups; therefore, augmentation of senescence markers with time in LBW rats may be related to superimposed ongoing tissue injury, associated with known effects of fetal program-

Hypertension is established in this model by 8–12 wk in males and increases after catch-up growth in humans (25, 26, 55). Early, subtle hypertensive injury likely contributes to acceleration of senescence, as has been shown in injured human kidneys and hypertensive rats (38, 57). Analogously, a kidney with a congenital nephron deficit likely undergoes hyperfiltration and thereby slowly accumulates injury although histological changes are often subtle (4, 21). Chronic glomerular/nephron stress therefore also likely contributes to ongoing renal senescence with age (34).

Catch-up growth is a known risk factor for accelerated cardiovascular disease and premature death (18, 44, 45). Catch-up growth in LBW rats is likely a critical factor contributing to early senescence, which is then further exacerbated by organ growth and adaptation. Variability in an individual rat’s growth rate may also be a contributor to the increased variability of gene expression observed in LBW rats. In addition, from our data, diet composition itself appeared to have an independent effect on p16 expression in NBW rats, unrelated to birth weight or rate of growth. This is consistent with a report where NBW mice fed a cafeteria diet died earlier than those fed a normal diet (45). In our LBW rats, however, this effect of diet was not evident. LBW rats on R diets did gain

Fig. 2. Expression of p16, p21, and p53 by real-time RT-PCR in kidneys and hearts of LBW and NBW rats at birth and weaning. Values are means ± SD fold-change relative to 15-mo-old control rat heart or kidney (to make data comparable among 3 genes); n = 4 rats/group. Note differences in y-axis scale between genes and organs. *P < 0.001 LBW vs. NBW.
more weight than those on the H diet, with p16 tending to be higher in those on R diets, possibly suggesting an independent effect of body size. However, the observation that renal p16 expression was higher in all LBW rats, despite their remaining smaller and eating the same diets as NBW rats, does suggest a developmentally programmed susceptibility to accelerated senescence in LBW rats. It is likely therefore that the effects of perinatal programming, catch-up growth, adult diet, and body size all act synergistically to modulate development and progression of senescence with time.

Expression of the other potential senescence markers, p21 and p53, was not significantly increased in LBW compared with NBW kidneys at 6 mo. These findings are consistent with a report in human kidneys, where expression of p16 mRNA correlated significantly with increasing age, whereas p21 and p53 expression did not (37). However, expression of both p21 and p16, but not p53, was significantly increased in kidneys of weaned LBW animals. p21 expression is known to be upregulated during replicative senescence (61). In our model, upregulation of p21 during rapid catch-up growth may represent increased cell turnover during this period, which then subsides when growth rate declines. Increased p16 expression is known to occur after senescence is established; therefore, persistence of increased p16 expression in the LBW kidneys at 6 mo may reflect ongoing senescence in response to stress or the persistence of earlier senescence. We therefore suggest that there may be two synergistic pathways operating to augment senescence in LBW kidneys: first is a burst of accelerated replicative senescence during the period of rapid catch-up growth, as shown by elevations in both p21 and p16 at weaning, followed by subsequent persistence of stress-induced senescence reflected by p16 and hypophosphorylated Rb.

The patterns of gene expression in hearts were similar to those found in kidneys at birth and weaning for both NBW and LBW animals. The increased expression of senescence markers in LBW hearts at weaning, i.e., before hypertension is known to develop, suggests a possible proximal role for accelerated senescence in the development of cardiovascular pathophysiology in this model. These data also indicate that accelerated senescence in LBW rats during catch-up growth is not restricted to the kidney and may be a more systemic contributor to premature cardiovascular aging. Consistent with these findings, Tarry-Adkins et al. (52, 54) have reported reduced telomere length in the aorta and pancreas of LBW rats allowed to catch up after birth compared with NBW rats maintained on a low-protein diet or controls. In addition, expression of p16 and p21, but not p53, was increased in the pancreas of 3-mo-old catch-up rats compared with controls (52).

Oxidative injury has been proposed as a unifying process underlying the perinatal origins of adult disease (8, 10). Generation of ROS is also well recognized in the pathogenesis of chronic kidney disease (48, 61). Oxidative stress is a factor known to result in telomere damage and acceleration of senescence (2, 12). Among children born small for their gestational age, a significant increase in markers of oxidative stress and a reduction in antioxidant activity have been shown at birth and in cohorts from 4–13 yr old (14, 17, 40). Similarly, in young
rats subjected to intrauterine undernutrition, increased oxidative stress was associated with impaired endothelium-dependent vasodilatation and elevated blood pressures (15, 32). These data suggest that intrauterine programming of vascular responsiveness through oxidative stress is a link to subsequent hypertension, but whether this may be due to increased arterial stiffness associated with premature senescence is not known. Expression of antioxidant enzymes has been examined in several tissues after catch-up growth with varying results, likely reflecting an altered balance between basal expression and the capacity to respond to oxidative injury rather than absolute changes (33, 52–54, 56).

Our finding of increased mitochondrial p66Shc and ER Ero1α expression in LBW kidneys at 6 mo is highly suggestive of increased ROS production in these tissues (3, 9). Whether this is a programmed effect or in response to injury is not yet clear. Conceivably, an increased need for protein synthesis during catch-up growth may necessitate increased Ero1α production, which produces ROS, and may provide another mechanistic explanation for tissue injury (20). A reduction in p66Shc is associated with increased longevity; therefore, the increase in our 6-mo LBW kidneys, associated with elevated senescence markers, is consistent with premature aging in these animals (39).

Fig. 6. Representative Western blot analysis of Rb protein expression in whole kidney nuclear extracts from 6-mo-old rats. The top band represents phospho-Rb (110–112 kDa), and the middle band represents hypophosphorylated Rb (107 kDa). Values are means ± SD of the ratio of densitometry of hypophosphorylated to phosphorylated Rb relative to actin. *P < 0.01 for pooled LBW vs. NBW kidneys; n = 4/group.
Mitochondrial abnormalities have been reported in rats subjected to gestational protein restriction followed by catch-up growth. Mitochondrial DNA content from liver and skeletal muscle was reduced in fetal and early postnatal protein-restricted rats, which persisted later in life (47). Similarly, a significant reduction in mitochondrial cofactor coenzyme Q9 content was found in kidneys of 12-mo-old rats that had undergone rapid postnatal weight gain (51). These authors also suggest that increased ROS production from dysfunctional mitochondria, coupled with a potential defect in antioxidant defenses, may be critical in contributing to DNA damage, accelerated senescence, and premature death in LBW animals after rapid catch-up growth.

Our study has several limitations. We have not measured blood pressures and markers of renal function in our rats. Although correlations would have been interesting and may have explained some of the increased variability within the LBW group, the absence of these parameters does not detract from our conclusions. It is possible that the different times of weaning between LBW and NBW rats may have introduced some confounding; however, renal p16 levels were higher in LBW rats at weaning than NBW rats at 3 mo (Fig. 3), and therefore the 1-wk difference is unlikely to have contributed greatly to the differences in renal p16 expression at weaning. The subsequent gene expression differences between birth weight groups at 3 and 6 mo are also unlikely to have been affected by time of weaning. The difference in age at weaning does, however, limit conclusions that can be drawn as to relative rates of change in weight between weaning and 6 wk. Surprisingly, rats on the H diet did not exhibit hyperphagia or develop obesity in either birth weight group. It is possible that the ratio of free fat mass to lean body weight, or energy expenditure, may have been higher in LBW rats; however, these were not measured. Induction of obesity, however, is not inevitable in rat models and appears variable even within rat strains (11, 45, 49). In mice in which low-protein diets were continued from gestation through lactation similar to our study, no catch-up growth was observed on a highly palatable cafeteria diet, suggesting a long-term programming effect on appetite (46).

In conclusion, expression of the senescence marker p16 is significantly increased in kidneys and hearts of LBW rats after a period of rapid catch-up growth. In kidneys, p16 expression increases with time and is associated with increased expression of p66Shc and Ero1α, suggesting increased ROS production. Whether a primary event, e.g., early growth restriction, and/or a second “hit” occurring later, e.g., accelerated catch-up growth, makes an organ more vulnerable to premature aging, via increased metabolic demands (small kidneys in larger bodies), physical injury (hyperfiltration as a result of reduced nephron mass), or other extrinsic injury (e.g., programmed hypertension or diabetes), remains to be elucidated. Developmentally programmed accelerated senescence may be an important link between low birth weight and the risk of adult disease.

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![Fig. 8. Representative Western blot analysis of Ero1α protein expression in whole kidney cytoplasmic extracts from 6-mo-old rats. Values are means ± SD expressed as the ratio of Ero1α relative to actin as averaged from 2 blots. *P < 0.001 for pooled LBW vs. NBW kidneys; n = 4/ group.](http://ajprenal.physiology.org/)

![Fig. 7. Expression of mitochondrial stress marker p66 by real-time RT-PCR in kidneys of LBW and NBW rats at birth, weaning, and 6 mo of age. Values are means ± SD of p66 as % HPRT. †P < 0.03 LBW vs. NBW, birth. *P < 0.02, pooled LBW vs. NBW 6 mo; n = 4–8 rats/group.](http://ajprenal.physiology.org/)
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DISCLOSURES
No conflicts of interest are declared by the authors.

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