Naturally Occurring Perinatal Growth Restriction in Mice Programs Cardiovascular and Endocrine Function in a Sex- and Strain-Dependent Manner

ROBERT D. ROGHAIR AND GILBERT ALDAPE

Departments of Pediatrics, University of Iowa, Iowa, IA 52242

ABSTRACT: Perinatal growth restriction (PGR) is associated with type 2 diabetes and hypertension. Identification of an isogenic mouse model of fetal programming will facilitate mechanistic understanding. We sought to test the hypotheses that 1) PGR impairs glucose tolerance and induces hypertension; and 2) the programming phenotype is more severe in an inbred mouse strain susceptible to diabetes (C57BL/6) than in a strain without such genetic predisposition (DBA/2). PGR pups were paired at weaning with same sex normally grown littermates. Glucose and insulin tolerance tests were performed at 17 wk, and systolic blood pressure (SBP) was measured at 20 wk. Impaired glucose tolerance was evident only among female PGR mice from both strains. While PGR did not alter insulin sensitivity in either strain, female DBA/2 mice had significantly decreased insulin levels during glucose tolerance testing. SBP was increased in PGR male C57BL/6 mice ($p < 0.01$), while heart rate was decreased in PGR male DBA/2 mice ($p < 0.05$). These studies indicate that in isogenic mice, PGR alters endocrine and cardiovascular physiology in female and male mice, respectively. Strain- and sex-specific genetic susceptibilities emphasize the need to consider genetic predisposition when evaluating the role of the perinatal environment in the inception of adult disease. (*Pediatr Res* 62: 399–404, 2007)

Associations between perinatal growth restriction and adult disease first came to light following the seminal work of Dr. David Barker showing men with the lowest weights at birth and at one year went on to have the highest death rates from ischemic heart disease (1). His work has been followed by epidemiologic studies across diverse human populations that showed decreased birth weight is independently associated with an increased incidence of hypertension and diabetes in later life (2). Studies in a variety of animal species have also shown that maternal nutrient restriction, fetal glucocorticoid exposure or uterine artery occlusion can lower birth weight and induce development of the metabolic syndrome in adulthood (2). Although these studies have clearly defined patterns of adult disease, they use experimental interventions that do not completely encompass all the etiologic factors involved in naturally occurring growth restriction. Studies of naturally occurring birth weight variation within multiple gestation pregnancies have been completed in humans, pigs and rats (3–7). In humans, twin studies have demonstrated smaller

twins have increased blood pressure (3) and impaired glucose tolerance (4). Similarly, Poore *et al.* used a purebred pig model to show that adult mean arterial pressure (5) and serial glucose levels following glucose administration (6) were inversely correlated with birth weight. Finally, Woods *et al.* demonstrated that for outbred rats, adult blood pressures were significantly higher in low birth weight animals (7).

While each of these studies used birth weight as an entry criterion to investigate the “fetal origins of adult disease”, with the awareness that birth itself does not mark the end of mammalian development, the field has now expanded to fully encompass the breadth of the “developmental origins of health and disease” (2). Harkening back to the work of Dr. Barker (1), recent studies of a Helsinki birth cohort continue to lend support to this broader developmental perspective. These studies by Eriksson and colleagues show that low birth weight and low weight gain during infancy synergistically increase the risk of developing type 2 diabetes and coronary heart disease (8,9). While these studies demonstrate early events can permanently program adult physiology, an important role for genetic susceptibility to adult disease has also been demonstrated in both human and murine studies (10,11).

Given the commercial availability of inbred (isogenic) mouse strains of varying genetic susceptibilities, the ability to generate knockout and transgenic mice to test gene-specific mechanisms and the desire to limit experimental interventions during development, we sought to identify a naturally occurring programming model within an isogenic mouse strain. We chose to investigate the effects of growth restriction throughout the period of developmental immaturity, encompassing both fetal and early postnatal growth (to the age of weaning). We hypothesized that 1) perinatal growth restriction (PGR) leads to impaired glucose tolerance and hypertension; and 2) the programmed phenotype will be more severe in an inbred mouse strain susceptible to diabetes (C57BL/6) than a strain without such genetic predisposition (DBA/2).

METHODS

Animal model. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996) and was

Received January 31, 2007; accepted June 3, 2007.

Correspondence: Robert D. Roghair, M.D., Department of Pediatrics, Division of Neonatology, 8804 JPP, Children’s Hospital of Iowa, Iowa, IA 52242; e-mail: robert-roghair@uiowa.edu

This work was supported by grant K08HD050359 from the National Institutes of Health/National Institute of Child Health & Human Development.

Abbreviations: GTT, glucose tolerance test; ITT, insulin tolerance test; PGR, perinatal growth restriction; SBP, systolic blood pressure

approved by the University of Iowa Animal Care and Use Committee. Growth restricted mouse pups ("runts") from inbred C57BL/6 and DBA/2 strains were identified by breeding facility personnel (Charles River Laboratories, Wilmington, MA) at the time of weaning (postnatal d21). These naturally occurring growth-restricted pups (>2 standard deviations below mean litter weight) were immediately paired with a same sex normally grown littermate and then transferred to the University of Iowa Animal Care Unit. The mice were co-housed, weighed weekly, and provided standard 4 kcal/g rodent diet (7013; Harlan Teklad, Madison, WI). At 17 wk, the mice were separated to individual cages (to record feed intake over the final 3 wk of the study), and crown-rump length was measured. After a year of breeding, 25 pairs of mice were transferred ($n = 8$ pair male C57BL/6; 7 pair female C57BL/6, 5 pair male DBA/2 and 5 pair female DBA/2). One female growth restricted C57BL/6 pup died at 14 wk following postnatal weight gain of only 7g. This pup and her paired control were excluded from all calculations (final $n = 6$ pair female C57BL/6).

Glucose and insulin tolerance. At 17 wk, glucose tolerance tests and insulin tolerance tests were performed. For the glucose tolerance tests, mice were fasted 3h before tail snip blood collection for fasting glucose (One Touch Blood Glucose Meter, LifeScan Inc). At this same time, 50 μ L of blood was collected for fasting plasma insulin determination (Ultra sensitive rat/mouse insulin assay; Crystal Chem Inc). Sterile 20% dextrose was then administered by intraperitoneal injection (2 g/kg). Glucose measurements were repeated 30, 60 and 120 min after the glucose injection. At the 30-min time point, 30 μ L of blood was collected for plasma insulin determination. Following the fasting insulin assay, sufficient plasma volume was present from the C57BL/6 samples (but not the DBA/2 samples) to measure fasting leptin levels (Murine leptin assay, Diagnostic Systems Laboratories, Inc). After at least 48 h of recovery, insulin tolerance tests were performed in an analogous fashion. Mice were fasted for 3h before baseline glucose measurement. After intraperitoneal injection of regular human insulin (0.75 U/kg; Humulin-R, Eli Lilly, Indianapolis, IN), glucose measurements were repeated in 15, 30 and 60 min. These repeat glucose measurements were then normalized as a percent of baseline glucose.

Tail cuff blood pressure. At 20 wk, mice were acclimated on 4 consecutive days to an indirect tail-cuff systolic blood pressure (SBP) and heart rate apparatus (BP 2000; Visitech Systems, Apex, NC). SBP was then measured at a consistent time of day for an additional 4 d. A warming plate kept the mice at 38°C, and holding units were used. Automated tail cuffs placed proximally on the mouse's tail were rapidly inflated to occlude blood flow for 30 30-s cycles each day. On deflation, the return of blood flow was detected by a photoresistor below each tail and the corresponding SBP and heart rate were digitally recorded and subsequently averaged for each mouse. The tail cuff apparatus was unable to detect pulsatile blood flow in one female and one male PGR C57BL/6 mouse; these mice and their paired controls were excluded from SBP and heart rate analysis.

Data analysis. All values are presented as mean $\pm$ SEM. Statistical comparisons between PGR and control mice were performed by paired t test or linear regression, as appropriate. Statistical evaluation for sex- or strain-specific differences was performed using 2-way ANOVA with multiple comparisons evaluated by Tukey test. Serial weights and glucose values during glucose tolerance test (GTT) and insulin tolerance test (ITT) were compared by two way repeated measures ANOVA followed by Holm Sidak testing when ANOVA identified significant differences. A value of $p < 0.05$ was considered significant. All analyses were performed using SigmaStat 3.0 (SPSS Inc., Chicago, IL).

RESULTS

Growth and nutrition. By definition, PGR pups weighed significantly less than control pups at 3 wk (Fig. 1; all $p < 0.01$). PGR mice remained significantly smaller than control mice throughout the study (Fig. 1). For all mice, serial weights were no longer significantly different from the final adult weight by 13 wk. Weight at 17 wk (final weight before invasive testing) was significantly reduced in both male and female PGR mice from either strain (Table 1), but adult length was significantly decreased only among PGR male mice (Table 1; C57BL/6: $p < 0.01$; DBA/2: $p < 0.05$). Sexual dimorphism was present for both adult weight and length among control C57BL/6 mice, but not DBA/2 mice (Table 1). Daily caloric intake was significantly decreased in male PGR

Figure 1. Perinatal growth restricted mice were paired with same sex littermates at 3 wk. Male growth restricted (■) and control (□) mice from C57BL/6 (A; $n = 8$) and DBA/2 (C; $n = 5$) strains, as well as female growth restricted (●) and control (○) mice from C57BL/6 (B; $n = 6$) and DBA/2 (D; $n = 5$) strains were weighed weekly for 17 wk. * $p < 0.05$ or ** $p < 0.01$ or † $p < 0.001$ vs control by two way repeated measures ANOVA.

C57BL/6 mice ($92 \pm 2\%$ of control, $p = 0.01$), but not female PGR C57BL/6 mice ($102 \pm 2\%$ of control) or either sex of PGR DBA/2 mice (male: $85 \pm 8\%$ of control; female: $86 \pm 7\%$ of control, both $p = 0.13$). When normalized for weight at 18 wk, PGR female C57BL/6 mice consumed significantly more calories per kg than their paired controls (Fig. 2; $p < 0.05$).

Glucose and insulin tolerance. While fasting glucose levels before GTT were lower in the entire study population of PGR mice (PGR: 8.2 ± 0.3 mM; control: 9.0 ± 0.3 mM; ANOVA $p < 0.01$), post hoc analysis did not show significant differences within individual groups (Table 1). Similarly, fasting plasma leptin and insulin levels were not significantly altered by PGR (Table 1). Regardless of strain, response to glucose administration was unaffected by PGR in male mice (Fig. 3A and 3C). In contrast, glucose administration induced significantly higher peak glucose levels in female PGR mice from either strain (Table 1). By repeated measures ANOVA, glucose levels were consistently elevated in female PGR DBA/2 mice (Fig. 3D). Among those female PGR DBA/2 mice, this glucose intolerance was associated with significantly lower insulin levels 30 min following glucose administration (Table 1).

Fasting glucose levels before ITT were not significantly altered by PGR (Table 1). Of note, both PGR and control male C57BL/6 mice had lower fasting glucose levels before GTT than they had before the subsequent ITT ($p < 0.01$). Following insulin administration, glucose levels declined similarly in PGR and control mice, whether analyzed as a percentage of fasting glucose (Fig. 4) or without normalization (data not shown). Compared with male DBA/2 mice, male C57BL/6

Table 1. Weight, length, leptin, insulin and glucose levels measured at 17 weeks

	C57BL/6				DBA/2			
	Male (n = 8)		Female (n = 6)		Male (n = 5)		Female (n = 5)	
	PGR	Control	PGR	Control	PGR	Control	PGR	Control
Weight, g	22.7 ± 0.8**	26.3 ± 0.6¶	19.5 ± 0.7*	21.2 ± 0.3	20.9 ± 1.6**	25.2 ± 1.8	21.5 ± 0.8*	23.4 ± 0.6
Length, cm	9.4 ± 0.1**	10.0 ± 0.2§	9.1 ± 0.1	9.4 ± 0.1	9.6 ± 0.2*	10.2 ± 0.2	9.8 ± 0.2	9.9 ± 0.2
Fasting Leptin, ng/ml	4.7 ± 0.4	5.5 ± 0.5§	4.0 ± 0.1	3.9 ± 0.3				
Fasting Insulin, mM	123 ± 13	142 ± 21§	74 ± 17	69 ± 15	139 ± 54	164 ± 30	88 ± 11	139 ± 34
Fasting Glucose-GTT, mM	8.2 ± 0.7	8.6 ± 0.7	8.5 ± 1.2	8.9 ± 1.0	7.5 ± 0.8	8.5 ± 0.7	7.1 ± 0.2	7.3 ± 0.3
Peak Glucose-GTT, mM	17.0 ± 1.8	17.0 ± 1.4‡	14.4 ± 0.6*	11.3 ± 0.8	8.7 ± 0.8	9.9 ± 1.1	10.3 ± 0.7*	8.2 ± 0.5
Insulin 30 min into GTT, mM	135 ± 10	156 ± 24	141 ± 23	107 ± 18	66 ± 56	105 ± 32	48 ± 6*	84 ± 12
Fasting Glucose-ITT, mM	9.9 ± 0.4	10.6 ± 0.4‡	9.5 ± 0.7	9.6 ± 0.5	7.6 ± 0.6	8.3 ± 0.3	7.7 ± 0.5	8.1 ± 0.8

Values are mean ± SEM; GTT, glucose tolerance test; ITT, insulin tolerance test; PGR, perinatal growth restriction.

* $p < 0.05$ or ** $p < 0.01$ versus control.

‡ $p < 0.01$ versus DBA/2.

§ $p < 0.05$ or ¶ $p < 0.01$ versus female.

Figure 2. Growth restricted (solid bars) and paired control mice (open bars) were separated into individual cages at 17 wk. Intake of standard rodent feed was recorded over the following 3 wk and normalized to weight at 18 wk. * $p < 0.05$ vs control.

mice had higher peak glucose levels during GTT and higher fasting glucose levels before ITT (Table 1). Compared with female C57BL/6 mice, male C57BL/6 mice had significant elevations in fasting leptin, fasting insulin and peak glucose during GTT (Table 1).

Blood pressure and heart rate. Overall, PGR was associated with higher adult blood pressures, with the greatest differences seen in male C57BL/6 mice (Fig. 5A; male C57BL/6, $p < 0.01$; female C57BL/6, $p = 0.08$; male DBA/2, $p = 0.14$; female DBA/2, $p = 0.25$). By linear regression (Fig. 6A), weight at 3 wk (time of weaning) was inversely related to adult systolic blood pressure for C57BL/6 mice ($p < 0.05$), but not DBA/2 mice ($p = 0.32$). In contrast, PGR was associated with lower heart rate during tail cuff blood pressure recording in male DBA/2 mice (Fig. 5B; $p < 0.05$). Similarly, by linear regression, weight at 3 wk was directly related to adult heart rate for DBA/2 mice ($p < 0.01$), but not C57BL/6 mice ($p = 0.79$).

DISCUSSION

The ability of early life events to program adult disease became evident following demonstration of a strong inverse

Figure 3. Glucose levels were measured during intraperitoneal glucose tolerance testing at 17 wk for male growth restricted (■) and control (□) mice from C57BL/6 (A; $n = 8$) and DBA/2 (C; $n = 5$) strains, as well as female growth restricted (●) and control (○) mice from C57BL/6 (B; $n = 6$) and DBA/2 (D; $n = 5$) strains. Values are displayed as means with vertical lines indicating SEM. * $p < 0.05$ vs control by two way repeated measures ANOVA.

correlation between birth weight and risk of cardiovascular and endocrine disorders, including hypertension and type 2 diabetes (12). Although animal models have subsequently shown that these adult disorders can also be programmed by intrauterine exposures in the absence of perinatal growth restriction, perinatal growth restriction remains the most easily identifiable marker for a neonate at risk of developing adult disease (2). We therefore sought to demonstrate that naturally occurring perinatal growth restriction programs murine development in a fashion analogous to that described in other species. We used two isogenic mouse strains commonly used for knockout and transgenic studies to evaluate the role of genetic susceptibility and define the most representative

Figure 4. Insulin sensitivity was measured during intraperitoneal insulin tolerance testing in male growth restricted (■) and control (□) mice from C57BL/6 (A; $n = 8$) and DBA/2 (C; $n = 5$) strains, as well as female growth restricted (●) and control mice (○) from C57BL/6 (B; $n = 6$) and DBA/2 (D; $n = 5$) strains. Data are normalized to baseline glucose levels. There were no significant differences between growth-restricted and control mice.

model for human disease. Our results demonstrate that natural perinatal growth variation is strongly associated with adult cardiovascular and endocrine programming. We further show that both genotype and gender play important roles in the ultimate pattern of adult disease.

In this study, we categorized mice as growth restricted based on their weight at the time of weaning. While it is not

possible to define whether these mice were growth restricted as a result of an adverse fetal and/or neonatal environment, this time point was chosen in consideration of emerging epidemiologic data and comparative mammalian developmental trajectories. For example, unlike precocious species, such as humans and sheep, that complete nephrogenesis weeks before delivery, mice and rats are altricial species (immature at birth) that continue nephrogenesis through the first week of life (13). Related to these varying windows of susceptibility, antenatal dexamethasone administration permanently programs sheep blood pressure only when given early in gestation (14), while rats can be programmed by such interventions from the end of gestation through the early neonatal period (15,16).

Regardless of strain, PGR mice remained smaller than their paired littermates throughout the study with male mice having the greatest impairment in adult weight and length. This perinatal constraint of postnatal growth is consistent with the direct relationship between birth weight and adult stature seen in the prospective human twin studies of Loos *et al.* (17,18). Among the factors that regulate postnatal growth, there is strong evidence for perinatal programming of postnatal appetite (19). We likewise noted increased daily caloric intake specifically among female PGR C57BL/6 mice. This was not associated with significant alteration in leptin levels, but we are unable to exclude the possibility of acquired leptin resistance. Other factors that can be affected by perinatal influences and can themselves influence postnatal growth, including changes in body composition and locomotor activity also require further investigation (20).

Female PGR mice from both inbred mouse strains had impaired glucose tolerance despite preservation of insulin sensitivity. This glucose intolerance was most dramatic among female DBA/2 mice that had significantly reduced insulin

Figure 5. Tail cuff systolic blood pressure (A) and heart rate (B) were recorded at 20 wk for growth restricted (solid bars) and paired control mice (open bars). * $p < 0.05$ and ** $p < 0.01$ vs control values.

Figure 6. Correlation between weight at 3 wk and either systolic blood pressure (SBP; A) or heart rate (B) at 20 wk for C57BL/6 mice ($n = 24$, □, dashed line) and DBA/2 mice ($n = 20$, ●, solid line). Pup weight was inversely correlated with SBP for C57BL/6 mice (A; $r = 0.41$, $p < 0.05$) and directly correlated with heart rate for DBA/2 mice (B; $r = 0.57$, $p < 0.01$).

levels during GTT, suggesting the glucose intolerance they demonstrated may be related to a deficit in stimulated insulin secretion. This proposed mechanism is consistent with the findings of Jensen and colleagues showing former small for gestational age young adults have a 30% decrease in stimulated insulin secretion (21) before the development of the insulin resistance commonly seen in programmed metabolic syndrome (2). Of note, all DBA/2 mice had a paradoxical reduction in plasma insulin concentration during glucose tolerance testing. While unexpected, it is possible this reduction was due to pulsatile insulin release, limited pancreatic reserves for additional insulin secretion or strain-specific reliance on insulin independent pathways, including increases in motor activity or IGF 1. Further studies with additional hormone levels are thus necessary to better define this programmed phenotype.

As expected, there were dramatic inter-strain differences in both fasting glucose and glucose tolerance, with C57BL/6 mice having significantly elevated glucose levels. This relative glucose intolerance seen in C57BL/6 mice has recently been linked to inbred defects in stimulated insulin secretion (22). Our studies further demonstrate that relative to DBA/2 mice, C57BL/6 mice have impaired insulin sensitivity. This combination of impaired insulin secretion and insulin resistance is reminiscent of human type 2 diabetes, and suggests the C57BL/6 strain is a relevant model for further investigation of the links between perinatal exposure and adult metabolic syndrome.

The strong inverse relationship between pup weight and systolic blood pressure seen in the C57BL/6 mice is likewise consistent with the findings seen in both epidemiologic studies and diverse animal models (2). The presence of a significant effect in male mice is consistent with previously published data (15,23,24). Of note, these sex-specific differences in the pattern of fetal programming mirror the sexual dimorphism seen in control animals. That is, while male C57BL/6 control mice are glucose intolerant (22), it is the female mice that became so following perinatal growth restriction; and while female C57BL/6 control mice are hypertensive (25), it is the male mice that became so following perinatal growth restriction. It therefore appears that alterations in perinatal growth manifest quiescent phenotypes more than they exaggerate preexisting physiologic differences. Along these lines, it is notable the SBP obtained by Deschepper and colleagues in 9 wk C57BL/6 mice (male: 114 ± 3 mm Hg, $n = 10$; female: 129 ± 2 mm Hg, $n = 10$) (25) were comparable to our control data (male: 120 ± 3 ; female: 130 ± 8 mm Hg), and lower than our PGR data (male: 137 ± 4 ; female: 138 ± 9 mm Hg). It is also important to note that our study was not powered to detect less than a 10 mm Hg difference in SBP, and we may have failed to detect a true difference in SBP between PGR and control C57BL/6 female mice.

The mechanisms responsible for the programmed hypertension seen following perinatal growth restriction are not known, but may be related to impaired nephrogenesis, increased sympathetic drive and altered endothelial-dependent vascular reactivity (2,26,27). A role for exaggerated stress responses is supported by relative increase in the second set of

fasting glucose levels obtained for C57BL/6 mice. While the cause of this increase in fasting glucose may be related to a variety of factors, including difference in the ambient environment, it is also possible there were stress-induced alterations in neuroendocrine status either as a learned response to the prior GTT or as a physiologic response to the elevated glucose levels during the previous testing.

The lack of correlation between perinatal growth restriction and blood pressure in the DBA/2 mice was unexpected. The absence of hypertension may be related to a highly significant decrease in heart rate among the perinatal growth restricted animals. The etiology of the decreased heart rate in the PGR DBA/2 mice remains undefined, with possible contributions from changes in baroreceptor activity, central sympathetic drive and the overall stress response of the PGR mice. It should therefore be noted that the use of tail cuff methodology requires animal restraint and thereby engenders animal distress. Although indirect blood pressure measurements are not stress-free in any animal species, we attempted to minimize the stress our mice experienced using experienced handlers and daily acclimation. While we attempted to minimize experimental interventions as much as possible throughout this study, future investigations may benefit from the use of implanted radiotelemeters to better evaluate cardiovascular parameters during rest, activity, and times of psychological or physiologic stress.

Utilizing an isogenic mouse strain with genetic predisposition toward cardiovascular and endocrine disease (C57BL/6), we have demonstrated that naturally occurring perinatal growth restriction blunts the sexual dimorphism normally present by inducing the development of hypertension in male mice and glucose intolerance in female mice. In mice from an isogenic strain resistant to the development of the metabolic syndrome (DBA/2), perinatal growth restriction similarly programs adult cardiovascular and endocrine function in a sex-specific fashion. Genetic and environmental manipulations of these inbred mouse strains should complement research being conducted in alternative species as the search for the cause and treatment of an important subset of adult diseases progresses.

Acknowledgments. The authors thank Dr. Curt Sigmund for facilitating use of the tail cuff monitoring apparatus.

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