Transgenerational effects of prenatal nutrient restriction on cardiovascular and hypothalamic-pituitary-adrenal function

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The perinatal environment is a powerful determinant of risk for developing disease in later life. Here, we have shown that maternal undernutrition causes dramatic changes in heart structure and hypothalamo-pituitary-adrenal (HPA) function across two generations. Pregnant guinea pigs were fed 70% of normal intake from gestational days 1–35 (early restriction; ER), or 36–70 (late restriction; LR). Female offspring (F_1) were mated and fed *ad libitum* to create second generation (F_2) offspring. Heart morphology, blood pressure, baroreceptor and HPA function were assessed in male F_1 and F_2 offspring. ER_{F1} males exhibited elevated blood pressure, increased left ventricular (LV) wall thickness and LV mass. These LV effects were maintained in the ER_{F2} offspring. Maternal undernutrition increased basal cortisol and altered HPA responsiveness to challenge in both generations; effects were greatest in LR groups. In conclusion, moderate maternal undernutrition profoundly modifies heart structure and HPA function in adult male offspring for two generations.

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There is now considerable evidence that cardiovascular disease has its origins in prenatal life (Hanson & Gluckman, 2005). Epidemiological studies have demonstrated that size at birth is related to subsequent risk of Type 2 diabetes, insulin resistance, hypertension, cardiovascular disease and stroke (Barker *et al.* 1993). These relationships are strong, supported both by clinical investigation and experimental research (Hanson & Gluckman, 2005). Studies of human populations following famine have suggested that pathologies in later life are dependent on the timing of nutritional insult during pregnancy. Follow-up of the Dutch Hunger Winter cohort showed that cardiovascular disease was more prevalent in offspring of mothers who were severely undernourished during the first trimester of their pregnancies in 1944/5 compared to those born to mothers whose pregnancies were more advanced at the time of nutritional insult (Roseboom *et al.* 2001*a*).

A number of mechanisms by which the early environment can influence cardiovascular function have been proposed. These include changes in the renin–angiotensin system (Dodic *et al.* 1998), expression of glucocorticoid-inducible genes (Gardner *et al.* 1998; Bertram *et al.* 2001), vascular smooth muscle properties (Bendeck *et al.* 1994) and endothelial function (Torrens *et al.* 2003). The origins of these relationships remain largely unknown, but maternal exposure to stress or elevated glucocorticoids have been suggested as important causative factors. Recent studies in guinea pigs, rats and sheep suggest that exposure to suboptimal uterine environments caused by physiological insults such as restraint, poor nutrition, hypoxia or elevated glucocorticoid exposure can induce changes in hypothalamic-pituitary-adrenal (HPA) axis function which persist throughout postnatal life (McCormick *et al.* 1995; Levitt *et al.* 1996; Liu *et al.* 2001; Lingas & Matthews, 2001; Sloboda *et al.* 2002; Lesage *et al.* 2002). Long-term alterations in HPA function have been linked to premature development of adult onset pathologies including insulin resistance and hypertension (Nyirenda *et al.* 1998; Reynolds *et al.* 2001; Welberg & Seckl, 2001; Matthews, 2002).

The guinea pig represents a good model for investigation of susceptibility to programming stimuli at specific time

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windows since, like humans, this species gives birth to relatively mature offspring (Dobbing & Sands, 1970, 1979; Owen & Matthews, 2003; Owen *et al.* 2005). While gestation length in the guinea pig is long for a small mammal (70 days), it is considerably shorter than that of larger mammals (e.g. primates and sheep), making transgenerational studies feasible.

There is emerging evidence that developmental effects induced by an environmental stimulus are not limited to the first generation (Drake & Walker, 2004). However, in epidemiological studies it is difficult to determine the relative importance of genomic and non-genomic effects of the pre- and postnatal environment, against heterogeneous backgrounds of genetic susceptibility. The impact of any effects in different populations will vary, making it difficult to assign cause to any one environmental factor. In contrast, animal studies provide strong support for the hypothesis that non-genomic intergenerational effects operate across a number of generations. Such effects have been demonstrated for insulin resistance and hepatic glucose regulation in the rat model (Drake *et al.* 2005; Zambrano *et al.* 2005, 2006). However, no studies have reported transgenerational effects on cardiovascular or HPA function. In the present study, we assessed the effect of timed-restricted nutrition during pregnancy on the cardiovascular system and HPA axis of guinea pig offspring, and ascertained whether effects were transmitted to the second generation in the absence of an additional nutritional challenge.

Methods

Animals and experimental treatments

All studies described were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. The protocols were also approved by the Ethics Committee of the University of Southampton. Nulliparous 3- to 4-month-old female Dunkin Hartley guinea pigs ($n = 46$; Halls Farm, Shropshire, UK) were mated as previously described (Banjanin *et al.* 2004). Briefly, onset of oestrus was defined as the time of vaginal membrane rupture. At this time, females were placed with one of four unrelated breeding males. After mating, guinea pigs were assigned to one of three groups, early restriction (ER, $n = 15$), late restriction (LR, $n = 14$) and control (C, $n = 17$), and housed in individual cages under 12 : 12 h light–dark conditions in visual, auditory and olfactory contact with other pregnant guinea pigs. Standard guinea pig/rabbit chow (Special Diet Services, Witham, UK: 3.4% fat, 18.5% protein, 10.2% cellulose, 9.4% ash, 0.33% methionine) was fed. In addition, all animals had free access to tap water supplemented with vitamin C (400 mg l^{-1}, guinea pigs, like humans, are unable to produce vitamin C). Food-restricted animals were fed between 08.30 and 09.30 h daily. Animals on a restricted diet were given 70% of the dietary provision of the control group for either days 1–35 (ER) or days 36–70 (LR) of pregnancy. Daily intake was calculated using a small pilot study of pregnant guinea pigs under control conditions. During gestation, maternal weight was recorded twice weekly and food intake measured daily. At birth, litter size, weight, nose–rump and abdominal circumference of all pups were recorded. First generation C_{F1} ($n = 10$ female, $n = 9$ male), ER_{F1} ($n = 15$ female, $n = 18$ male) and LR_{F1} ($n = 22$ female, $n = 18$ male) offspring were weaned at 21 days of age, at which point males were housed individually. Non-sibling F_1 females were used to generate the F_2 generation. Where there was more than one female in a litter, the female that had a birth weight closest to the mean weight of the litter was used (C_{F1}, $n = 7$; ER_{F1}, $n = 7$; LR_{F1}, $n = 11$). Animals were group housed until their second oestrus and mated with control males. F_1 females were not subjected to any food restriction. Their offspring are referred to as C_{F2} ($n = 10$ female, $n = 9$ male), ER_{F2} ($n = 7$ female, $n = 10$ male) and LR_{F2} ($n = 18$ female, $n = 22$ male). Animals were left undisturbed apart from daily animal care and bi-weekly weight measurement.

At 90 days of age, polyvinyl catheters (PE90, Royem Scientific, Luton, UK) were surgically implanted into a carotid artery and a jugular vein of non-sibling male offspring (C_{F1} $n = 8$, ER_{F1} $n = 7$, LR_{F1} $n = 8$, C_{F2} $n = 6$, ER_{F2} $n = 6$, LR_{F2} $n = 6$), as previously described (Liu *et al.* 2001). Briefly, anaesthesia was induced by halothane inhalation (4% in O_2), and maintained by isofluorane (1.5–2.0% in O_2) and surgery was carried out under standard aseptic conditions. Local anaesthetic (lidocaine (lignocaine) hydrochloride, Astra Zeneca, UK) was administered prior to closure of the skin to minimize post-operative discomfort. A small guinea pig jacket with an integral spring was fitted, through which the arterial catheter was passed to attach to a Teflon swivel (Lomir Biomedicals, Montreal, Canada). This allowed full rotation of the catheter and unrestricted movement of the guinea pig. Analgesic (buprenorphine 0.1 mg kg^{-1} I.M., Schering-Plough Ltd, UK) was given before the anaesthetic was discontinued and 24 h following surgery. Catheters were filled with heparinized saline and flushed daily. Animals were allowed to recover for a minimum of 5 days following surgery. We have shown that repeated sampling of animals catheterized in this way does not result in activation of the HPA axis (Liu & Matthews, 1999; Liu *et al.* 2001).

Cardiovascular analysis

Prior to surgery, cardiac function and morphology was assessed in conscious adult F_1 and F_2 males using echocardiography (by an operator blinded to treatment

group) to measure the left ventricular wall (LVW) thickness, left ventricular mass (LVM) and fractional shortening (FS; a measure of cardiac function) using previously validated techniques. LVW thickness was measured using a Sonos 2500 scanner with 5 or 7.5 MHz linear transducers, and images taken from the left or right parasternal windows in either prone or right lateral decubitus position. Short axis views at the level of the tip of the mitral valve were used to obtain M mode targeted recordings. Left ventricular diastolic and systolic dimensions were measured using the leading edge technique. FS and LVM were calculated using the equations:

$$\mathrm{FS} = (\mathrm{LVD} - \mathrm{LVS})/\mathrm{LVD} \times 100$$

and

$$\mathrm{LVM} = 1.055\{\mathrm{LVD} - \mathrm{LVW[d]} + (\mathrm{IVS[d]})^3 - (\mathrm{LVD})^3\}$$

where LVD is left ventricular wall diameter in diastole, LVS is left ventricular wall diameter in systole and IVS is inter-ventricular septal wall thickness. Baseline blood pressure and heart rate were measured 8 days post-surgery via the carotid artery catheter, using a small displacement pressure transducer connected to a MacLab/4e (AD Instruments, Chalgrove, UK) data acquisition system and using MacLab Chart 4.5.6 software. Blood pressure was measured continuously between 09.00 and 12.00 h. Chart analysis was performed using the same software to calculate systolic, diastolic and mean arterial pressure (MAP) and heart rate. Following this baseline recording, a bolus of phenylephrine (1.0 mg kg^{-1}) was administered I.V. to measure both the sensitivity of the arterial vasculature to α-adrenergic stimulation, and also the set-point and gain of the baroreflex. The gain of the baroreflex was derived from the plot of blood pressure *versus* heart rate, as the slope of the linear portion of the plot; set-point of the reflex was taken as the blood pressure at which the mid-point between baseline heart rate and that following the phenylephrine bolus occurred.

Hypothalamo-pituitary-adrenal analysis

At 95 days of age, animals were challenged with dexamethasone (DEX) and corticotrophin-releasing hormone (CRH) to assess glucocorticoid feedback sensitivity and pituitary–adrenal responsiveness to activation by CRH, respectively. DEX (1 mg kg^{-1}, I.V.; Sigma, Poole, UK) was administered at 09.00 h. Blood samples (250 μl per time point) were taken just prior to DEX administration (−30 and 0 min) and at +30, 60, 120 and 240 min following injection. This was followed by an injection of human CRH (0.5 μg kg^{-1}, I.V.; Sigma, Poole, UK) at 13.00 h (240 min) and continued blood sample collection at 245, 255, 270, 300 and 360 min. The doses of DEX and CRH used in the current study were derived from our previous studies (Liu & Matthews, 1999; Liu *et al.* 2001; Banjanin *et al.* 2004). All sampling was done out of the line of sight of the animals, to reduce the possibility of stress to the animal at the time of drug administration/blood removal.

Double-antibody and coated tube radioimmunoassay (RIA) kits (ICN Biomedical Inc., Belgium) were used to determine plasma concentrations of adrenocorticotrophin (ACTH) and cortisol, respectively. These assays have been previously used in the guinea pig (Liu *et al.* 2001; Owen & Matthews, 2003). All samples from within each test were run in the same assay to eliminate interassay variability. Intra-assay coefficients of variance for ACTH and cortisol were 4.9% and 5.2%, respectively.

At the end of the sampling protocol, animals were killed with an overdose of pentobarbital (I.V.), immediately decapitated and trunk blood taken. The brains, pituitary, adrenals, livers and kidneys were removed and weighed. For each animal, the right hippocampus was dissected and weighed.

Statistical analysis

All data were expressed as mean ± S.E.M. For all tests, significance was set at $P < 0.05$. Statistical analysis was performed using multivariate analysis of variance (ANOVA), followed by Duncan's method of *post hoc* comparison or a Bonferroni *post hoc* test to compare replicate means. Gestation length, litter size, birth weight, blood pressure, heart rate, organ weights, body weights, organ-to-body/brain weight ratios, were analysed by one-way (prenatal treatment) ANOVA with two-sample *t* tests to test specific hypotheses. The growth data were analysed by repeated-measures ANOVA.

For analysis of treatment differences, ACTH and cortisol data were divided into DEX suppression (0–240 min) and CRH activation (240–360 min) periods and analysed using one-way (treatment) ANOVA on the integrated net area above (DEX suppression, AAC) or under (CRH activation, AUC) the curve. AAC was calculated using net change in cortisol or ACTH concentration between basal values at 0 (prior to DEX administration) and values at 240 min using the trapezoidal rule. Similarly, AUC was calculated using net change from values at 240–360 min.

Results

Physiological measurements

In the first generation, prenatal nutritional restriction had no effect on gestation length (C_{F1} 69.1 ± 0.3 days, ER_{F1} 69.2 ± 0.3 days, LR_{F1} 69.1 ± 0.4 days). ER_{F1} females, but not males, were significantly heavier than controls at birth

(Fig. 1). Longitudinal analysis across all time points in Fig. 1 showed increased absolute growth in ER_{F1} females ($P < 0.04$) but not males. In contrast, for LR_{F1}, birth weight and growth were reduced in both females ($P < 0.003$) and males ($P < 0.05$). In the second generation, birth weight and growth in ER_{F2} did not differ from that of the controls. However, birth weight in the LR_{F2} females was significantly reduced. Longitudinal analysis across all time points showed reduced absolute growth in both LR_{F2} females ($P < 0.02$) and LR_{F2} males ($P < 0.05$). LR_{F1} females took significantly ($P < 0.01$) longer to conceive than C_{F1} or ER_{F1} females (age at pregnancy: C: 85 ± 13.7 days, ER 92.7 ± 13.6 days, LR 164.8 ± 11.8 days), although this delay was not associated with either changes in age or in weight at 1st oestrous (Table 1). Maternal body weight during pregnancy is shown in Table 2. There was considerable variability within groups, probably a result of the fact that guinea pigs carry 2–4 fetuses per litter and these each weigh approximately 100 g at birth.

Organ to body weight ratios for male offspring from the different prenatal treatment groups (both generations) are presented in Table 3. In the F_1 generation, there was a significant decrease in pituitary-to-brain and adrenal-to-body weight ratios in both the ER_{F1} and LR_{F1} animals ($P < 0.01$). These corresponded to significant decreases in both pituitary and adrenal weights. Kidney- and brain-to-body weight ratios were significantly increased in the ER_{F2} and LR_{F2} groups. Liver to body weight ratio was higher in the LR_{F2} group compared to the controls (C_{F2}); however, this appears to be related to a reduction in the C_{F2} liver to body weight ratio rather than an absolute increase in the LR_{F2}. In contrast, there were significant reductions in relative adrenal weight in the LR_{F2} (but not ER_{F2}) group, and although there were no differences in absolute brain weight, there were reductions in the hippocampal and pituitary weights (expressed as a proportion of brain weight) in both the ER_{F2} and LR_{F2} groups.

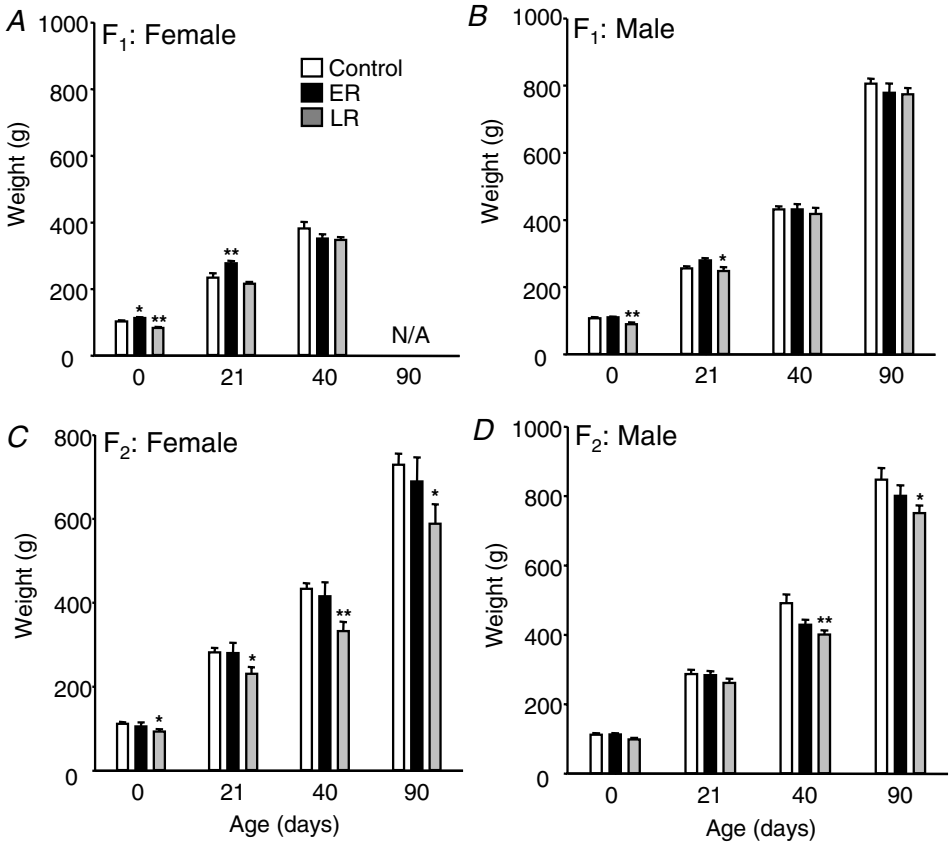

Figure 1. Birth weight and growth
Animal weights at birth and at 21 and 40 days (mean ± S.E.M.) in F_1 females (*A*) and at birth and 21, 40 and 90 days of age in F_1 male (*B*), F_2 female (*C*) and F_2 male (*D*) offspring born to control mothers (male F_1 $n = 9$, F_2 $n = 9$; female F_1 $n = 10$, F_2 $n = 10$) or mothers that had been nutritionally restricted (30%) during early (ER; gestational days 1–35; male F_1 $n = 18$, F_2 $n = 10$; female F_1 $n = 15$, F_2 $n = 7$) or late (LR; gestational days 36–70; male F_1 $n = 18$, F_2 $n = 22$; female F_1 $n = 22$, F_2 $n = 18$) pregnancy. $^*P < 0.05$, $^{**}P < 0.01$ denote significant differences compared with controls. N/A, data not available as F_1 females were mated after day 40.

Table 1. Reproductive function

	Age (days)			Body weight (g)			Successful mating (oestrous)			
	First oestrous	First mating	Pregnancy	First oestrous	First mating	Pregnancy	2nd	3rd	4th	5th
C_{F1}	41.7 (1.7)	56.7 (1.9)	85.0 (13.7)	389 (27.5)	479 (28.4)	692 (55.5)	3	2	1	1
ER_{F1}	43.3 (2.6)	54.0 (4.5)	92.7 (13.6)	376 (18.1)	494 (21.7)	565 (55.2)	2	4	1	0
LR_{F1}	42.0 (1.8)	54.7 (1.5)	164.8 (11.8)**	372 (14.5)	452 (31.4)	794 (26.4)**	0	4	4	3

Age and weight at first oestrous and mating, together with body weight and mating outcome, in F_1 generation female offspring whose mothers were nutritionally restricted during early (ER; $n = 7$) or late (LR; $n = 11$) pregnancy or fed *ad libitum* (control, C; $n = 7$). Group data (mean ± S.E.M.) were analysed using one-way analysis of variance (ANOVA) and Bonferroni *post hoc* comparison. $^{**}P < 0.01$ denotes significant difference compared with controls.

Table 2. Weight (g) during pregnancy of F_0 and F_1 females at different stages of gestation (days)

	10	20	30	40	50	60	70
C_{F0} ($n = 17$)	714.3	747.0	782.6	856.6	940.9	1054.9	1136.9
	(32.2)	(33.3)	(30.4)	(31.4)	(32.6)	(36.4)	(37.5)
ER_{F0} ($n = 15$)	645.1	678.5	713.8	804.9	933.8	1051.9	1133.8
	(44.0)	(45.4)	(46.1)	(48.9)	(53.9)	(58.7)	(60.3)
LR_{F0} ($n = 14$)	703.1	738.3	789.3	864.0	964.5	1058.2	1140.2
	(38.9)	(40.2)	(34.7)	(34.4)	(36.5)	(40.9)	(42.1)
C_{F1} ($n = 7$)	750.8	787.9	834.0	898.1	1002.8	1098.9	1138.9
	(71.8)	(63.9)	(67.4)	(49.5)	(45.2)	(57.8)	(53.7)
ER_{F1} ($n = 7$)	634.6	687.8	728.7	816.0	927.7	1023.2	1068.6
	(44.0)	(38.1)	(38.1)	(32.5)	(38.3)	(45.8)	(42.6)
LR_{F2} ($n = 11$)	840.6	864.6	888.7	955.1	1054.8	1134.0	1171.5
	(27.2)	(23.1)	(23.3)	(27.9)	(30.3)	(34.6)	(32.1)

Comparison of weight gain of F_0 and F_1 females through pregnancy. F_0 females were nutritionally restricted during early (ER_{F0}) or late (LR_{F0}) pregnancy or fed *ad libitum* (C_{F0}). These F_0 females were the mothers of the F_1 females (F_1 females were fed *ad libitum* throughout pregnancy). Group data (mean ± S.E.M.) were analysed using one-way analysis of variance (ANOVA) and Bonferroni *post hoc* comparison.

Table 3. Organ weights in male offspring

	Percentage of body weight					Percentage of brain weight	
	Liver	Kidney	Adrenal	Brain	Brain weight (g)	Hippocampus	Pituitary
C_{F1}	3.6 (0.3)	0.42 (0.03)	0.035 (0.001)	0.58 (0.01)	4.2 (0.07)	4.3 (0.33)	0.47 (0.03)
ER_{F1}	3.3 (0.1)	0.44 (0.01)	0.031 (0.001)**	0.60 (0.01)	4.3 (0.03)	4.1 (0.33)	0.41 (0.04)**
LR_{F1}	3.2 (0.1)	0.38 (0.01)	0.026 (0.001)**	0.58 (0.01)	4.3 (0.06)	4.2 (0.35)	0.43 (0.03)**
C_{F2}	2.9 (0.2)	0.33 (0.01)	0.039 (0.001)	0.57 (0.01)	4.7 (0.10)	4.3 (0.23)	0.43 (0.02)
ER_{F2}	3.1 (0.1)	0.41 (0.01)**	0.041 (0.001)	0.63 (0.02)**	4.7 (0.08)	3.8 (0.41)**	0.39 (0.08)
LR_{F2}	3.4 (0.1)**	0.43 (0.01)**	0.033 (0.001)**	0.62 (0.01)**	4.7 (0.06)	4.0 (0.30)*	0.40 (0.02)**

Organ weights as percentage of body weight (brain, adrenal, liver and kidney) and hippocampal or pituitary weights as a percentage of brain weights in adult offspring whose mothers or grandmothers had been subjected to nutritional restriction during either days 1–35 of gestation (ER_{F1} ($n = 7$) and ER_{F2} ($n = 6$)) or days 36–70 (LR_{F1} ($n = 8$) and LR_{F2} ($n = 6$)) compared to control animals (C_{F1} ($n = 8$) and C_{F2} ($n = 6$)). Results are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ denote significant difference compared with controls of the same generation.

Cardiovascular function

Heart rate and blood pressure. In the F_1 generation males, MAP was significantly ($P < 0.05$) elevated in the ER_{F1} compared to control and LR_{F1} groups (Fig. 2*A*). There were no significant differences in heart rate between prenatal treatment groups (C_{F1} 325 ± 6, ER_{F1} 324 ± 9, LR_{F1} 322 ± 10 beats min^{-1}). In the F_2 generation males, neither MAP (Fig. 2*A*) nor heart rate (data not shown) were different between groups.

Echocardiography. In the F_1 generation males, IVS ($P < 0.05$), anterior left ventricle wall (LVW; $P < 0.01$) and mean LVW ($P < 0.01$) thicknesses were significantly greater in ER_{F1} than in C_{F1} or LR_{F1} groups (Fig. 2*B*). There was no difference in fractional shortening (FS; a measure of cardiac function) between the groups (Table 4), but the corrected left ventricular (LV) mass of ER_{F1} animals was significantly ($P < 0.05$) increased compared with the C_{F1} and LR_{F1} groups. In the F_2 generation males, septal wall ($P < 0.05$), anterior LVW ($P < 0.05$) and mean LVW thicknesses ($P < 0.05$) were also significantly greater in ER_{F2} animals compared to C_{F2} and LR_{F2} groups (Fig. 2*C*). As with the F_1 generation, there was no significant difference in FS between groups, but the corrected LV mass was significantly ($P < 0.05$) higher in the ER_{F2} group (Table 4).

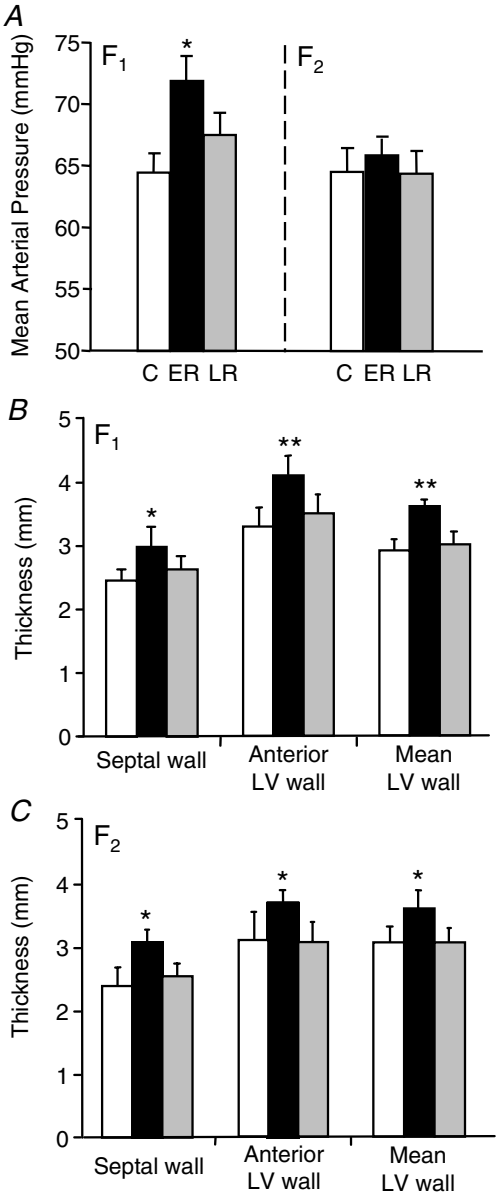

Figure 2. Cardiovascular function
A, mean arterial pressure (mean ± S.E.M.) in adult F_1 and F_2 male offspring whose mothers (F_1) or grandmothers (F_2) had been subjected to 30% reduction in total food intake during early (ER; black bars; F_1 $n = 7$, F_2 $n = 6$) or late (LR; grey bars; F_1 $n = 8$, F_2 $n = 6$) pregnancy or in offspring from control mothers (C; white bars; F_1 $n = 8$, F_2 $n = 6$). Echocardiography: interventricular septal wall thickness, anterior left ventricular wall thickness and mean left ventricular (LV) wall thickness (mean ± S.E.M.) in adult F_1 (*B*) and F_2 (*C*) male offspring in the same groups of animals. $^*P < 0.05$, $^{**}P < 0.01$ denote significant differences compared with controls of same generation.

Baroreflex responses. The baroreflex response sensitivity, in terms of the prolongation of R–R interval induced by elevation of blood pressure following a phenylephrine bolus, was significantly greater ($P < 0.05$) in the LR_{F1} males compared to the control (C_{F1} 0.021 ± 0.002, ER_{F1} 0.025 ± 0.004; LR_{F1} 0.036 ± 0.007 ms mmHg^{-1}). In addition, the set-point on the blood pressure axis of the baroreceptor response curve in the ER_{F1} males was significantly reduced ($P < 0.05$) compared to controls (C_{F1} 95.0 ± 1.9, ER_{F1} 86.4 ± 2.7; LR_{F1} 91.8 ± 2.6 mmHg). There were no significant differences in baroreflex responses between groups in the F_2 generation males.

Hypothalamo-pituitary-adrenal function

Basal plasma ACTH concentrations were not significantly different between prenatal treatment groups in the F_1 generation males but were significantly increased in LR_{F2} males compared to the ER_{F2} and C_{F2} groups (Fig. 3*A* and *B*). However, in the F_1 and F_2 generations, basal plasma cortisol concentrations were significantly elevated in the ER and LR males compared to controls (Fig. 3*C* and *D*).

In the F_1 generation, administration of DEX caused a significant suppression of plasma ACTH concentrations (C_{F1} $P < 0.001$; ER_{F1} $P < 0.005$; LR_{F1} $P < 0.005$; Fig. 4*A*). Analysis of the net area above the curve (AAC) following suppression by DEX treatment revealed that both ER_{F1} and LR_{F1} animals exhibited a significantly reduced overall suppression of plasma ACTH concentrations compared to control animals. Following CRH injection, ER_{F1} animals exhibited a similar peak response to the controls, but because of the higher baseline following DEX suppression, the incremental change in ACTH concentrations was lower when analysis of the net AUC was undertaken (Fig. 4*A*). However, the LR_{F1} group exhibited a substantially increased plasma ACTH response whether assessed incrementally or absolutely (Fig. 4*A*). Administration of DEX caused a significant suppression of plasma cortisol concentrations (C_{F1} $P < 0.001$; ER_{F1} $P < 0.005$; LR_{F1} $P < 0.005$; Fig. 5*A*). Net AAC analysis of the cortisol response following DEX administration showed significantly greater cortisol suppression in ER_{F1}

Table 4. Echocardiography

	Control		ER		LR	
	F_1 ($n = 8$)	F_2 ($n = 6$)	F_1 ($n = 7$)	F_2 ($n = 6$)	F_1 ($n = 8$)	F_2 ($n = 6$)
LV diameter systole (mm)	8.75 (0.3)	8.28 (0.5)	8.80 (0.3)	7.90 (0.2)	8.62 (0.2)	7.81 (0.2)
LV diameter diastole (mm)	11.57 (0.3)	11.47 (0.4)	11.95 (0.3)	10.80 (0.2)	11.42 (0.1)	10.44 (0.1)
Fractional shortening	24.61 (1.1)	25.12 (1.4)	25.51 (1.3)	26.67 (1.9)	24.07 (1.7)	25.49 (0.9)
Corrected LV mass (mg g^{-1})	4.27 (0.2)	4.22 (0.3)	5.93 (0.3)*	5.30 (0.1)*	4.15 (0.3)	3.82 (0.2)

Biometric and cardiovascular data at 90 days of age in male offspring whose mothers (F_1) or grandmothers (F_2) had been subjected to 30% nutritional restriction during either days 1–35 of gestation (ER) or days 36–70 (LR) compared to control animals. Results are expressed as mean ± S.E.M. *$P < 0.05$ denotes significant difference compared to controls of same generation. LV, left ventricle.

and LR_{F1} animals compared to C_{F1} (Fig. 5*A*), although the post-DEX level of cortisol suppression measured at 120 and 240 min remained significantly higher in the LR_{F1} animals than the controls. Following CRH stimulation, AUC analysis revealed that the ER_{F1} group exhibited a significantly reduced plasma cortisol response compared with the other groups (Fig. 5*A*).

In the F_2 generation, DEX administration led to significant suppression of plasma ACTH levels (C_{F2} $P < 0.001$; ER_{F2} $P < 0.005$; LR_{F2} $P < 0.005$; Fig. 4*B*). Analysis of the plasma ACTH suppression (AAC) by DEX revealed a substantially increased net suppression in the LR_{F2} group of animals. Analysis of the net ACTH response (AUC) following CRH injection revealed very dramatic increases in the ACTH responses of both the ER_{F2} and LR_{F2} animals compared to controls (Fig. 4*B*). Administration of DEX led to significant suppression of cortisol (C_{F2} $P < 0.001$; ER_{F2} $P < 0.005$; LR_{F2} $P < 0.005$; Fig. 5*B*). Plasma cortisol concentrations at 09.00 h were significantly elevated ($P < 0.05$) in LR_{F2} and ER_{F2} animals compared to C_{F2}, and AAC analysis of the cortisol response to suppression by DEX administration showed a significant increase in overall cortisol suppression in ER_{F2} and LR_{F2} animals compared to C_{F2} (Fig. 5*B*). AUC analysis, however, revealed no overall differences in the level of plasma cortisol following activation by CRH in either ER_{F2} or LR_{F2} when compared to C_{F2} (Fig. 5*B*).

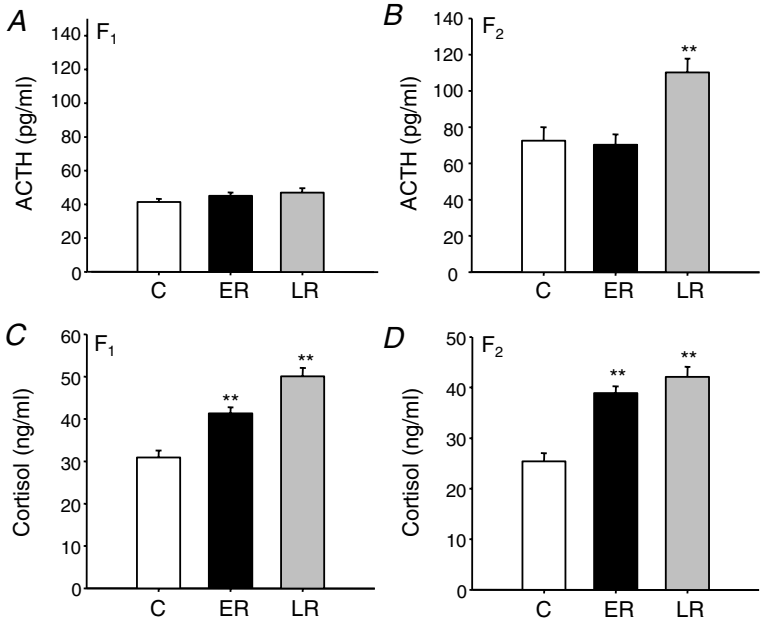

Figure 3. Basal HPA function
ACTH (*A* and *B*) and cortisol (*C* and *D*) concentrations (mean ± S.E.M.) in adult F_1 and F_2 male offspring whose mothers or grandmothers had been subjected to 30% reduction in total food intake during early (ER; gestational days 1–35; F_1 $n = 7$, F_2 $n = 6$) or late (LR; gestational days 36–70; F_1 $n = 8$, F_2 $n = 6$) pregnancy or in controls (C; F_1 $n = 8$, F_2 $n = 6$). **$P < 0.01$ denotes significant differences compared with controls of same generation.

Discussion

We have demonstrated that reduced nutrition during pregnancy has profound long-term effects on specific aspects of cardiovascular and HPA function, and that these effects are dependent on the timing of insult. Importantly, this study also shows that effects remain in the second generation offspring, despite no manipulation of the F_1 pregnancy.

Females exposed to early restriction had higher birth weights and postnatal growth, while both males and females exposed to late undernutrition had reduced birth and postnatal weights. These findings are intriguing as they accord with data from a human famine study. In the Dutch Hunger Winter Study, females who as fetuses were exposed to famine during the first trimester of pregnancy tended to have higher body weights, while those exposed in the last trimester had lower body weights; there was little effect in males (Ravelli *et al.* 1999). Strikingly, in the present study, an even greater effect of nutrient restriction in late gestation was identified in the F_2 generation, despite no manipulation during their development. The late restricted F_2 generation are also characterized by hypercortisolaemia and it is likely that their growth restriction is, at least in part, a consequence of glucocorticoid-mediated inhibition of growth, although other mechanisms may be involved, for example epigenetic modification of genes that regulate growth.

LR_{F1} females had a marked delay in conception which was not a result of altered body weight. This resulted in pregnant LR_{F1} females being twice the age of the C_{F1} and ER_{F1} females at conception. This novel observation may have parallels with the increasing body of human evidence that prenatal growth restriction results in reduced ovarian and uterine size, reduced fraction of primordial follicles and anovulation in late adolescence (Ibáñez *et al.* 2000).

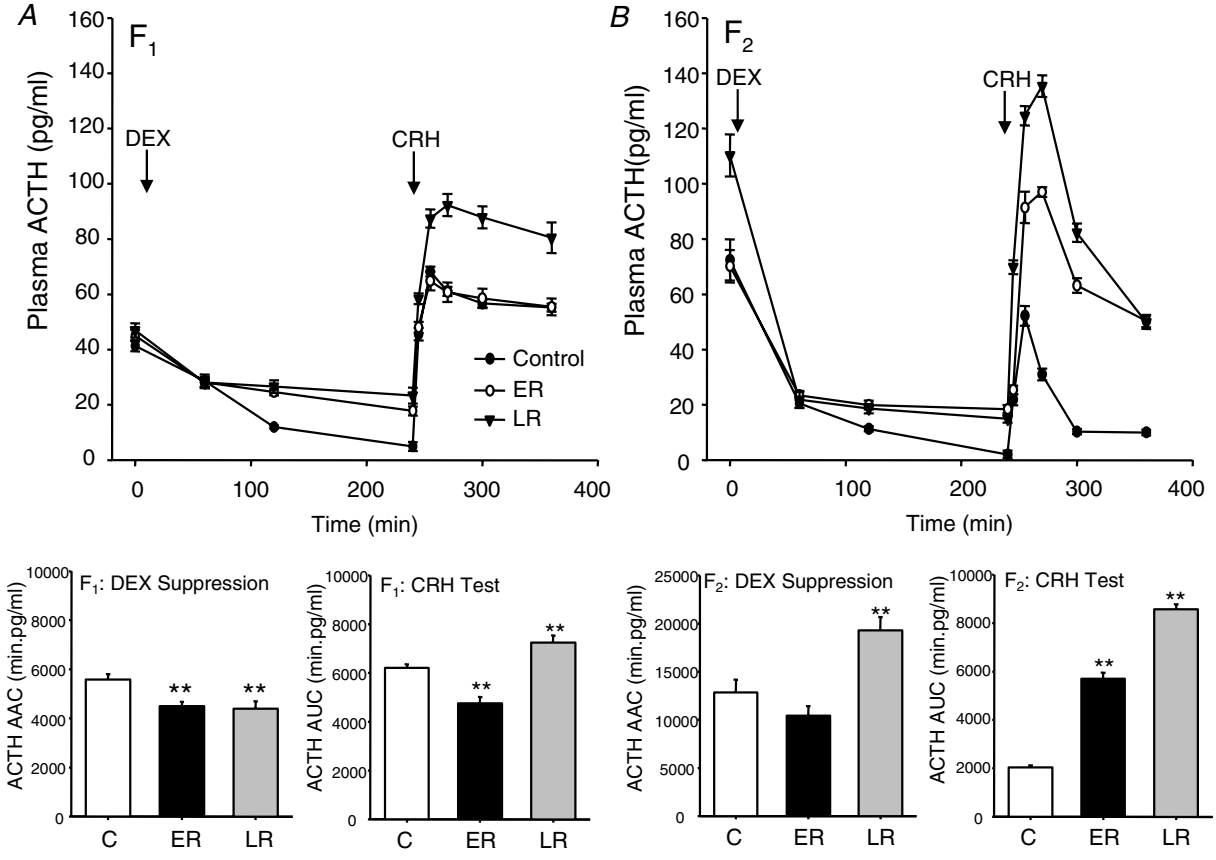

Figure 4. Dexamethasone suppression and corticotrophin-releasing hormone challenge (ACTH)
Plasma ACTH concentrations (mean ± S.E.M.) following dexamethasone (DEX, 1 mg kg^{-1}) and corticotrophin-releasing hormone (CRH, 0.5 μg kg^{-1}) challenge in adult male offspring whose mothers (*A*, F_1) or grandmothers (*B*, F_2) had been subjected to 30% reduction in total food intake during early (ER; gestational days 1–35; F_1 $n = 7$, F_2 $n = 6$) or late (LR; gestational days 36–70; F_1 $n = 8$, F_2 $n = 6$) pregnancy or in controls (C; F_1 $n = 8$, F_2 $n = 6$). $^{**}P < 0.01$ denotes significant differences compared with controls of the same generation. Histograms represent net area above the curve (AAC) of plasma ACTH concentrations after suppression by DEX and incremental area under the curve (AUC) plasma ACTH after activation with CRH.

Another apparent difference between the pregnant LR_{F1} females and the C_{F1} and ER_{F1} females was the reduced body weight gain during pregnancy in the pregnant LR_{F1} females. This is most probably related to age at pregnancy, as these animals were approximately 70 days older at the time of pregnancy than the C_{F1} and ER_{F1} groups. While guinea pigs are sexually mature at approximately 8 weeks of age they are still growing at this time. Therefore, part of the increase in weight during pregnancy in the C_{F1} and ER_{F1} groups is due to normal growth. In contrast, the older LR_{F1} animals are likely to have achieved adult body weight prior to pregnancy. We had not anticipated the reduced conception rate in the LR_{F1} and so were not able to factor differences in body weight into the experimental design. However, it is possible that maternal metabolic differences between the C_{F1} and ER_{F1} groups during pregnancy compared to the LR_{F1} group may impact on fetal development. Notwithstanding, our data illustrate more profound influences of nutritional restriction on cardiovascular and endocrine phenotype in ER_{F2} offspring compared to LR_{F2} offspring. Measurement of endocrine and nutrient levels in the blood of the F_1 dams would have given us additional mechanistic insight. However, catheterization or venepuncture are stressful, and we have previously shown that stress during pregnancy has profound effects on the offspring (Kapoor & Matthews, 2005). We did not make such measurements because the aim of this study was to examine the effect of suboptimal maternal nutrition on the offspring, and the results would have been confounded by additional stress.

The increase in MAP that we identified in the ER_{F1} offspring was associated with increased IVS and anterior LVW thickness. There was no growth restriction at any time in these offspring. In contrast, the LR_{F1} offspring

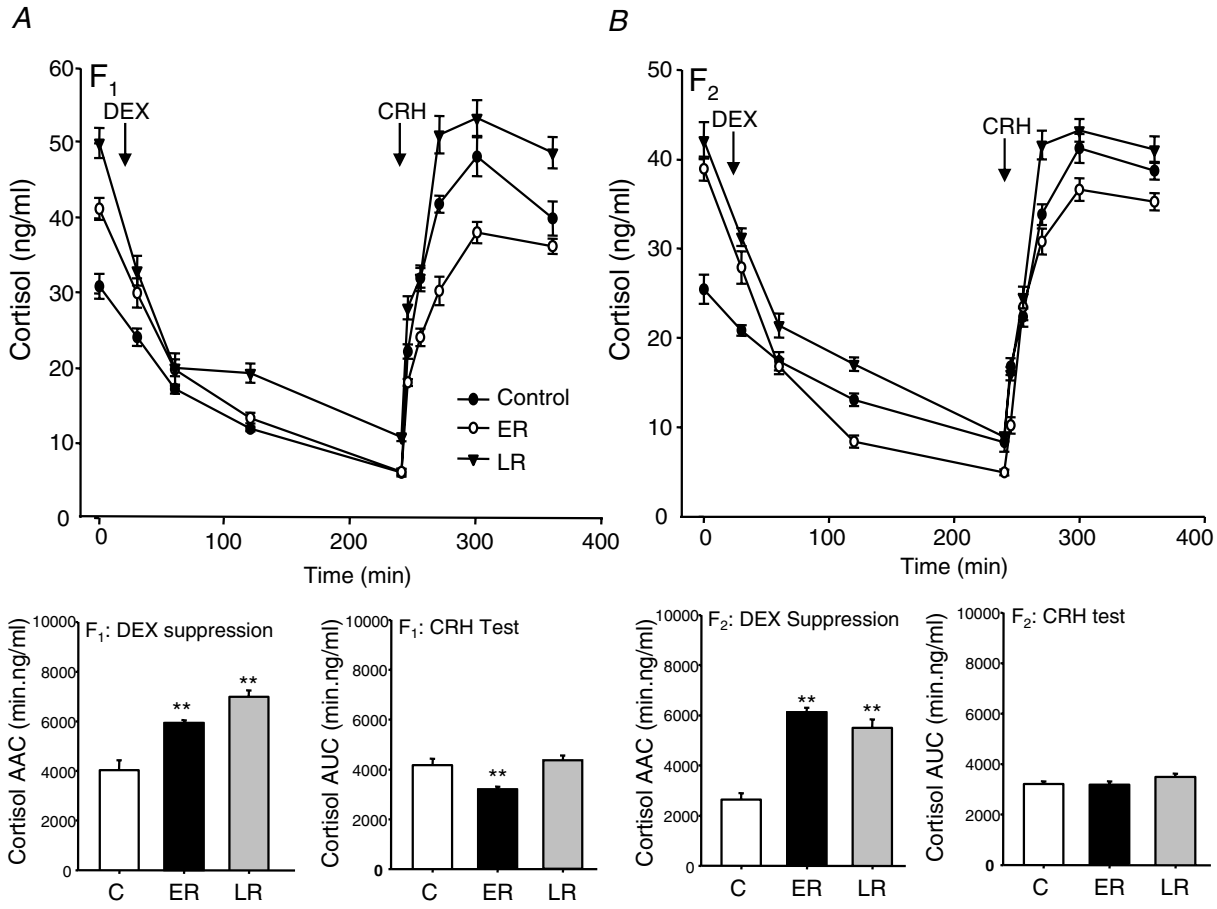

Figure 5. Dexamethasone suppression and corticotrophin-releasing hormone challenge (cortisol)
Plasma cortisol concentrations (mean ± S.E.M.) following dexamethasone (DEX, 1 mg kg^{-1}) and corticotrophin-releasing hormone (CRH, 0.5 μg kg^{-1}) challenge in adult male offspring whose mothers (*A*, F_1) or grandmothers (*B*, F_2) had been subjected to 30% reduction in total food intake during early (ER; gestational days 1–35; F_1 $n = 7$, F_2 $n = 6$) or late (LR; gestational days 36–70; F_1 $n = 8$, F_2 $n = 6$) pregnancy or in controls (C; F_1 $n = 8$, F_2 $n = 6$). $^{**}P < 0.01$ denotes significant differences compared with controls of the same generation. Histograms represent net area above the curve (AAC) of plasma cortisol concentrations after suppression by DEX and incremental area under the curve (AUC) plasma cortisol after activation with CRH.

were growth restricted but did not display alterations in blood pressure or LV structure. Clearly there is no direct relation between reduction in fetal growth and these cardiovascular effects. This accords with the idea that reduced growth is not on the causal pathway to later cardiovascular dysfunction, although it may be part of an adaptive strategy adopted by the developing organism (Hanson & Gluckman, 2005). Likewise, effects on cardiac development, or endothelial function leading to alterations in blood pressure, may represent distinct strategies which are induced to give later adaptive advantage (Hanson & Gluckman, 2005). The induction of these effects by an early gestation challenge has important implications for humans, since retrospective studies of the Dutch Winter Famine have indicated that adults whose mothers were exposed to famine in the first trimester of pregnancy had increased risk of cardiovascular disease (Roseboom *et al.* 2001*b*). In sheep, global undernutrition during the first half of pregnancy leads to increased fetal LVM by mid-gestation (Vonnahme *et al.* 2003).

In the F_2 generation, male offspring of ER_{F1} mothers demonstrated a strikingly similar LV enlargement to that in the ER_{F1} males, although this was not accompanied by elevated blood pressure. Therefore, the increased LV thickness in ER_{F2} animals is not a response to increased blood pressure. These findings clearly indicate that prenatal undernutrition can induce changes in cardiovascular structure and function which are passed across generations. Such non-genomic transmission clearly merits further mechanistic investigation. In addition, it was only feasible in this study to examine transmission via the female F_1 lineage. Future studies are needed to determine if similar effects can be passed via the male F_1 lineage, and indeed whether they are greater in F_2 offspring from female F_1 and male F_1 crosses.

The mechanisms by which the early environment influences the developing fetal heart are not known. It is possible that maternal undernutrition causes increased growth of the fetal heart, either directly, or in response to changes in the fetal peripheral circulation. This may involve an increase in the number of cardiomyocytes (Lijnen & Petrov, 1999; Pham *et al.* 2003; Han *et al.* 2004). Alternatively, maternal undernutrition during early gestation may induce premature maturation of the myocardium, reducing the number of cardiomyocytes at birth and thus exposing the heart to greater stress and hence hypertrophic increase in cardiomyocyte size postnatally (Yang *et al.* 2001). Finally, it is possible that early nutrient restriction causes epigenetic modification of genes that mediate cardiac hypertrophy in response to load; this may not affect fetal cardiomyocyte size or number but would predispose to the development of hypertrophy postnatally (Kuznetsova *et al.* 2003).

In the present study, baroreflex sensitivity to phenylephrine was increased in LR_{F1} offspring, while baroreflex set-point was significantly reduced in ER_{F1} offspring. Prenatal nutritional challenge has been shown to modify baroreflex function in rats and sheep (Gardner *et al.* 2004; Pladys *et al.* 2004). Interestingly, Gardner also found a left-shift in baroreflex function in lambs whose mothers had been nutritionally challenged during the periconceptual period (Gardner *et al.* 2004). Baroreceptors are critical for adapting to immediate changes in blood pressure. The lower set-point in the ER_{F1} group may be viewed as a compensatory mechanism to reduce the elevated blood pressure in this group. Similarly, it is possible that the greater gain of the reflex in the LR_{F1} animals had successfully brought MAP back to control levels.

Previous studies have shown that manipulation of the prenatal environment can alter HPA function in young and adult offspring (Welberg & Seckl, 2001; Owen *et al.* 2005). However, this is the first to demonstrate that the effects of undernutrition on HPA function are also present in the F_2 generation. Prenatal nutrient restriction had no effect on basal plasma ACTH concentrations in the F_1 generation. However, in F_2, basal ACTH levels were very substantially increased in the LR but not the ER group. In contrast to ACTH, plasma cortisol concentrations were significantly elevated in the ER and LR offspring in both F_1 and F_2; the effect was greatest in the LR group. This would suggest a substantial increase in adrenal sensitivity to circulating ACTH; except for in the LR_{F2} offspring where both basal plasma ACTH and cortisol were elevated. Alternatively, there may be a reduction in glucocorticoid metabolism, possibly resulting from an increase in corticosteroid-binding globulin. In a previous study, an acute period (48 h) of nutrient withdrawal, at the time of maximal fetal brain growth, resulted in adult F_1 male guinea pig offspring that exhibited a reduction in basal plasma ACTH and cortisol concentrations (Lingas & Matthews, 2001). In contrast, chronic severe nutrient restriction (50%) during the perinatal period in the rat had no effect on basal ACTH or corticosterone concentrations in adult male offspring (Lesage *et al.* 2002). Together these studies suggest that the effects of nutrient restriction on HPA function in adult F_1 offspring are dependent on the severity and timing of the nutrient deprivation. In the present study, there was a reduction in adrenal to body weight ratio in the ER_{F1}, LR_{F1} and the LR_{F2} offspring indicating that hypercortisolaemia was not associated with adrenal hypertrophy or hyperplasia. Chronic elevation of glucocorticoids can result in a reduction in hippocampal volume in the rat and human (Sapolsky, 1999; Lupien & Lepage, 2001; Roy & Sapolsky, 2003). In the present study, there was a significant reduction in hippocampal weight relative to brain weight in the ER_{F2} and LR_{F2} offspring compared to controls. Further analysis of neurological function in these animals is clearly warranted.

DEX suppression followed by CRH challenge is used for clinical assessment in humans (Newport *et al.* 2004). Reduced suppression of plasma cortisol following DEX exposure represents a sensitive test for depression. This approach provides a reproducible analysis of HPA function across many species (Banjanin *et al.* 2004). In the present study, there was a reduction in suppression of ACTH following DEX treatment in both the ER_{F1} and LR_{F1} offspring. The anterior pituitary represents the major feedback site for DEX (Newport *et al.* 2004), and as such the reduced ACTH suppression may result from a reduction in pituitary glucocorticoid receptors (GR). Surprisingly, the reduced ACTH suppression was associated with a greater suppression of plasma cortisol in ER_{F1} and LR_{F1} offspring. However, the latter probably resulted from the fact that basal plasma cortisol levels were elevated in both groups. CRH challenge following DEX suppression activated pituitary–adrenal function. Interestingly, there was a reduced net response of ACTH and cortisol in the ER_{F1} offspring compared to controls. This would suggest a reduced pituitary sensitivity to CRH and a resultant reduction in pituitary–adrenocortical response.

There were very significant effects of maternal nutrient restriction on HPA reactivity to the DEX–CRH challenge in F_2 offspring. The increased suppression of plasma ACTH by DEX in the LR_{F2} offspring was probably a result of the significant elevation in basal ACTH. Interestingly, DEX treatment in control F_1 and F_2 offspring resulted in an almost total loss of ACTH in the circulation, whereas this was not the case in ER and LR offspring of both generations, further indicating reduced GR sensitivity at the anterior pituitary. The net suppression of cortisol following DEX treatment was >2-fold elevated in the ER_{F2} and LR_{F2} offspring compared to controls. In humans, similar super-suppression of cortisol by DEX has been identified in post-traumatic stress disorder (Fries *et al.* 2005). In this regard, future studies of stress-related behaviour in animal models following nutrient restriction are required. In the present study, CRH challenge resulted in 2- and 3-fold greater ACTH response in the ER_{F2} and LR_{F2} offspring compared to controls. This would suggest a dramatic increase in pituitary sensitivity to CRH. However, these elevations in the ACTH response to CRH were not reflected by increased cortisol secretion from the adrenal cortex. This may result from a reduction in adrenocortical sensitivity or a decrease in the bioactivity of ACTH isoforms being secreted from the pituitary. Alternatively, it is possible that plasma ACTH (following CRH treatment) is maximally driving the adrenal cortex, such that further elevation of ACTH, as in ER_{F2} and LR_{F2} offspring, can cause no further elevation of cortisol secretion (i.e. a 'ceiling' effect). The latter may be resolved by decreasing the concentration of exogenously administered CRH, and clearly further studies are required.

The mechanisms that underlie transgenerational programming of cardiovascular and HPA function remain to be determined. Two major possibilities exist. Altered endocrine function in the ER_{F1} and LR_{F1} females results in altered metabolic, cardiovascular or endocrine adaptations which are known to be critical for pregnancy; this in turn influences development of the fetus. Alternatively, transgenerational transmission may occur via persistent epigenetic modification of DNA. Recent studies have shown that the early environment can have dramatic influences on methylation and histone acetylation in specific gene promoters including the GR, in F_1 offspring (Weaver *et al.* 2004; Lillycrop *et al.* 2005). Furthermore, a recent study has indicated that these epigenetic effects can be transgenerationally transmitted (Burdge *et al.* 2007), in which it is shown for the first time that altered methylation of gene promoters for peroxisome proliferator-activated receptor-alpha (PPARalpha) and GR induced in the F_1 generation by protein restriction during pregnancy is transmitted to the F_2 generation. There is also emerging evidence for transgenerational transmission down the paternal as well as the maternal line (Drake & Walker, 2004; Gluckman *et al.* 2007). Future studies will be designed to investigate this possibility in our undernutrition model.

In conclusion, moderate global nutrient restriction during pregnancy has profound effects on growth, reproductive efficiency, cardiovascular system, baroreceptor function and the HPA axis, and the timing of insult profoundly influences outcome. We have also demonstrated, for the first time, that it is possible for the effects which maternal nutritional restriction have on the cardiovascular system and the HPA axis, manifested in the first generation, to be passed to the second generation in the absence of any further manipulation. This has clear implications for our understanding of the development of human disease in later life as well as its non-genomic transgenerational transmission.

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