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BRIEF REPORT

Effect of a low-protein diet during pregnancy on expression of genes involved in cardiac hypertrophy in fetal and adult mouse offspring

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Gene markers for cardiomyocyte growth, proliferation and remodeling were examined in mouse fetuses and adult male offspring exposed to maternal low-protein (LP) diet during pregnancy. Whole heart volume, measured by magnetic resonance imaging, was smaller in day 15 LP fetuses *v.* those from chow-fed dams (C), whereas heart volume was greater in adult LP *v.* C offspring. These LP offspring were hypertensive and had larger cardiomyocytes *v.* C animals. The mRNA levels of cyclin G1, a marker for cell growth, were lower in LP fetal hearts *v.* C hearts, but similar in the left ventricle of adult LP and C offspring. Opposite trends were found in brain natriuretic peptide levels (a marker of cardiac hypertrophy). Thus, maternal LP during pregnancy results in smaller fetal hearts and is accompanied by changes in expression of genes involved in cardiomyocyte growth, which are associated with cardiac hypertrophy and hypertension in adulthood.

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Introduction

Epidemiological studies have suggested that inadequate maternal nutrition during early life may set in motion processes that increase the risk of cardiovascular disease (CVD) later in life.[1] This might be due to changes in intrauterine availability of nutrients affecting organ and tissue growth, which may be adaptive in the short term but could also result in the development of disease in later life.[2] However, the effects of maternal nutrient restriction on the developing heart are less well defined.

During early fetal development, the growth of the heart occurs primarily through the proliferation of cardiomyocytes,[3] but at terminal differentiation this switches from hyperplasia to hypertrophy in late prenatal or early postnatal life.[4] In rodents, this sequence is evident in early development when there is a rapid increase in the number of binucleated cardiomyocytes associated with increasing mechanical load.[5] The cardiomyocytes then mature and do not divide further. Nevertheless, cardiomyocyte proliferation can still be observed, albeit rarely, in adult and aging hearts of rodents as part of a compensatory mechanism to regenerate tissue mass and function.[6] During early development, cardiomyocyte proliferation can be influenced by nutritional, humoral and hemodynamic factors.[7]

To date, there is little information on the molecular mechanisms involved in cardiomyocyte proliferation. The G1 phase cyclins, such as cyclin G1, have been linked to cardiomyocyte hypertrophy[8] and the transcription factor, E2F1, has also been reported to be involved for progression from the G1 to the S phase.[9,10] Thus, E2F1, G1 phase cyclins and cyclin-dependent kinases are present in cells undergoing division. As G1 is the only phase of the cell cycle that can be influenced by external stimuli,[8,11] measuring expression of these genes may indicate whether the developmental environment is affecting cardiomyocyte proliferation and its progression to cardiac hypertrophy. In addition, in order to determine whether proliferation may lead to cardiac remodeling, we assessed gene expression levels for brain natriuretic peptide (BNP) in adult and fetal heart tissues. BNP is secreted to a much lesser degree during fetal development than in adult life, but its expression in the heart is recognized as a marker of cardiac hypertrophy in response to stress.[12,13] We hypothesized that maternal nutrient restriction alters cardiac development by affecting expression of genes involved in cardiomyocyte growth, proliferation and remodeling.

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Method

Experimental protocol

MF1 mice were maintained under a 12-h light/dark cycle, at constant temperature (22 ± 2°C) with water and food available *ad libitum*. All animal procedures were in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the local ethics review committee. At 8 weeks of age, female mice were mated and the day of appearance of a vaginal plug was assigned as day 0 of gestation. These pregnant females were then randomly allocated to a standard chow diet (C; contains 180 g of casein/kg; $n = 29$ pregnant females) or a low-protein diet (LP; contains 90 g of casein/kg; $n = 28$ pregnant females). A subgroup of pregnant dams (C, $n = 11$; LP, $n = 10$) was killed on day 15 of pregnancy by cervical dislocation and the fetuses were removed by hysterotomy. The heart was dissected from each fetus and pooled for each dam. Another subgroup of pregnant dams (C, $n = 8$; LP, $n = 8$) was also killed on day 15 of pregnancy, but this time a representative fetus taken from either side of the mid-horn of the gravid uterus from each pregnant dam was collected and fixed whole in 4% paraformaldehyde in phosphate buffered saline at 4°C for 2 weeks and then used for magnetic resonance imaging (MRI) analysis. The remaining pregnant dams (C, $n = 10$; LP, $n = 10$) were allowed to give birth and were then returned to the C diet. The number of pups per litter was standardized to eight at birth and were also fed a C diet after weaning. Systolic arterial pressure was measured in adult male offspring by tail-cuff plethysmography, as described previously.[14] They have shown that blood pressure taken by the tail-cuff method was highly correlated with intra-arterial blood pressure measured by telemetry in unrestrained, unanesthetized mice. At 6 months of age, a subgroup of the male offspring was killed by cervical dislocation and their hearts were collected. In a subset of heart samples, the left ventricle (LV) was dissected, frozen in liquid nitrogen and stored at −80°C until analyzed for gene expression. The remaining heart samples were fixed in 10% formalin and embedded in paraffin for histological and immunohistochemical analysis. In another subgroup of male offspring, the hearts were collected for MRI analysis. These hearts were arrested in diastole by intraperitoneal injection of a cardioplegic solution (St Thomas No. 1 formulation) prior to dissection to standardize the phase of the cardiac cycle in which they would be analyzed. We focused on studying the male adult hearts to avoid the confounding effects of sex differences, especially those mediated by estrogen.[15]

Magnetic resonance imaging

MRI was carried out as previously described.[16,17] Briefly, 16 whole fetuses were embedded in a nuclear magnetic resonance tube (outer diameter 28 mm) using 1% agarose (Seakem) containing 2 mM gadolinium-diethylenetriamine penta-acetic anhydride as a paramagnetic contrast agent. Imaging was carried out using an 11.7-Tesla (500 MHz) vertical magnet (Magnex Scientific, UK) interfaced to a Bruker Avance spectrometer (Bruker Medical, Germany). A matrix size of $512 \times 512 \times 768$ (bandwidth: 130 Hz/pixel) at a field of view of $26 \times 26 \times 30$ mm achieved an experimental resolution of $51 \times 51 \times 39$ μm. Dissected adult hearts were prepared in a similar manner but imaged with a 9.4T magnet interfaced to a 400 MHz Varian Direct Drive console (Varian Inc., USA). A matrix size of $608 \times 608 \times 1408$ at a field of view of $26 \times 26 \times 50$ mm achieved an experimental resolution of $43 \times 43 \times 36$ μm.

Raw MRI data were zero-filled to 1024^3 points (for fetuses) and to $1024 \times 1024 \times 2048$ points (for adult hearts) and reconstructed into a stack of 1024 out of 2048 2D TIFF files with 16-bit pixel resolution (for 16 fetuses, in which C $n = 8$ and LP $n = 8$, and for 20 adult hearts, in which C $n = 10$ and LP $n = 10$) using purpose written *C*-software. The TIFF files were analyzed using VGStudio Max (Volume Graphics) and 3D reconstructions were performed using image segmentation editing to calculate total fetal heart volumes and total tissue volumes in the adult heart.

Gene expression analysis of the fetal and adult heart

RNA was extracted from pooled fetal hearts (C, $n = 11$; LP, $n = 10$) and the LV of the male adult offspring (C, $n = 10$; LP, $n = 10$) and converted to cDNA by standard methods. TaqMan real-time PCR was then performed for each sample using the ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) and Eurogentec qPCR™ Mastermix reagents (Eurogentec Ltd, UK). The PCR reactions were carried out with one cycle at 50°C for 2 min and 95°C for 10 min. This was followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Primers and probes were synthesized as follows, with probes labeled with the 6-FAM and TAMRA at the 5′and 3′ ends, respectively: cyclin G1 forward, 5′-TTGGCTTTGACACGGAGACA-3′; cyclin G1 reverse, 5′-GCTTCGCCTG TACCTTCATTTT-3′; cyclin G1 probe, 5′-TTTCCCTAG CTGTGAATTTACTGGACAGATTCTTGT-3′; E2F1 forward, 5′-GCATCTATGACATCACCAATGT-3′; E2F1 reverse, 5′-GCTGCCTAGCCACTGGATATG-3′; E2F1 probe, 5′-TC CAGCTCATTGCCAAGAAGTCCAAGA-3′; BNP forward, 5′-GCTGTAACGCACTGAAGTTGT-3′; BNP reverse, 5′-TCAAAGGTGGTCCCAGAGCT-3′; BNP probe, 5′-ACT CCAGTTTCTGACTCTGCCTGGGTCTCTTT-3′; β-actin forward, 5′-GCTGTAACGCACACCCAGA-3′; β-actin reverse, 5′-CACAGCCTGGATGGCTACGT-3′; β-actin probe, 5′-TTTGAGACCTTCAACACCCCAGCCAT-3′. These oligonucleotides were designed using the Primer Express™ (v1.0) software based on their published sequences obtained from the GenBank database. The results were expressed relative to β-actin, which was used as the housekeeping gene.

Histological and immunohistochemical analysis

The fixed and paraffin-embedded whole hearts were cut into 6-μm thick sections. One set of sections was stained with

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Table 1. *Fetal heart volume and male offspring body weights, SBP and heart volume from dams fed a C or LP diet*

Dietary group	C	LP	*P*-value
Postnatal day 1 body weight (g)	1.69 ± 0.02 ($n = 27$)	1.62 ± 0.02 ($n = 28$)	0.04
Adult body weight (g)	46.10 ± 1.18 ($n = 17$)	45.54 ± 2.14 ($n = 17$)	ns
SBP (mm Hg)	98.3 ± 0.3 ($n = 17$)	113.6 ± 0.8 ($n = 17$)	<0.001
Fetal whole heart volume (mm^3)	3.1 ± 0.1 ($n = 8$)	2.5 ± 0.1 ($n = 8$)	<0.001
Adult heart tissue volume (mm^3)	147.1 ± 4.4 ($n = 10$)	167.7 ± 8.1 ($n = 10$)	0.038
Cardiomyocytes (μm^2)	247.6 ± 3.7 ($n = 7$)	264.1 ± 1.6 ($n = 7$)	0.002

SBP, systolic blood pressure; ns, not significantly different; C, standard chow diet; LP, low-protein diet.
Values are means ± S.E.M.
Numbers in parenthesis are the sample size.

Sirius red and cardiomyocyte area was measured in the longitudinal plane from the sections in which intercalated discs could be identified in nucleated cardiomyocytes. In each section, 10 high power fields were randomly selected, and up to 80 cells were measured. Another set of sections was incubated overnight at 4°C with polyclonal rabbit antiserum against Ki 67 antigen (1:750; Abcam Laboratories). Ki 67 is a nuclear antigen present only in the nuclei of cycling cells. Swine anti-rabbit antibodies were used as secondary antibodies (Dako Co., CA, USA). Staining was detected using an avidin-biotin-peroxidase method kit (ELITE ABC; Vector Laboratories, CA, USA) and 3,3′-diaminobenzidine.

Data analysis

Each data point (i.e. $n = 1$) was from a representative fetus or adult male offspring from each dam except for the fetal hearts used in gene expression analysis, which were pooled for each dam. All data are expressed as mean ± S.E.M. and all statistical analysis was by Student's *t*-test. Significance was assumed if the $P < 0.05$.

Results

Body weight and systolic blood pressure

On postnatal day 1, LP offspring weighed less ($P = 0.04$) compared to the C group (Table 1). However, the body weight at 6 months of age was not significantly different between the groups. Systolic blood pressure was higher ($P < 0.001$) in the adult LP offspring compared to the C group (Table 1).

Morphological changes detected by MRI

MRI analysis of the fetal heart on day 15 of pregnancy revealed smaller heart volume ($P < 0.001$) in LP compared to the C group (Table 1). On the other hand, the adult LP offspring had greater heart volume compared to the C group ($P = 0.038$).

Gene expression in the fetal and adult heart

Cyclin G1 mRNA levels in LP fetal heart tissue were lower ($P < 0.01$) *v.* C fetal hearts (Fig. 1a). No difference in cyclin G1 mRNA levels was found in adult LP and C offspring LV. No differences were found between the LP and C in E2F1 mRNA levels in the fetal heart or in the LV of adult offspring (Fig. 1b). Fetal heart BNP mRNA expression was similar between LP and C fetuses (Fig. 1c). In the adult offspring LV, however, BNP mRNA expression was higher ($P = 0.004$) in the LP group compared to the C offspring.

Histological and immunohistochemical analysis

Cardiomyocytes in the LP adult hearts were larger ($P = 0.002$) compared to the C group (Table 1). Ki 67 antigen immunohistochemical analysis did not show any difference in the number of Ki 67-positive nuclei between the experimental groups, but these were only rarely detectable and were found mainly in the cardiac fibroblasts rather than the cardiomyocytes.

Discussion

This study used MRI for the first time to compare morphologically the fetal and adult hearts of murine offspring exposed to maternal LP diet during pregnancy, in conjunction with measurements of genes involved in cardiomyocyte proliferation, cardiac hypertrophy and remodeling. MRI analysis shows that the heart volumes in LP fetuses were smaller compared with fetuses from pregnant dams on chow (C) diet, and this was accompanied by a lower level of expression of the cyclin G1 gene but without any changes in the expression of BNP and E2F1 compared with the C fetuses. These LP offspring, however, on reaching adulthood, were shown to have increased cardiac tissue volume. This was accompanied by histological findings of increased cardiomyocyte size and elevated BNP expression in the LV of the LP adult offspring heart compared to the C group. However, the differences in the level of expression of the cyclin G1 and E2F1 genes between the C group and LP adult offspring were no longer found in the LV tissue.

Measuring the expression of genes associated with cardiomyocyte proliferation, cardiac hypertrophy and remodeling in heart tissues has provided some novel insights into the early

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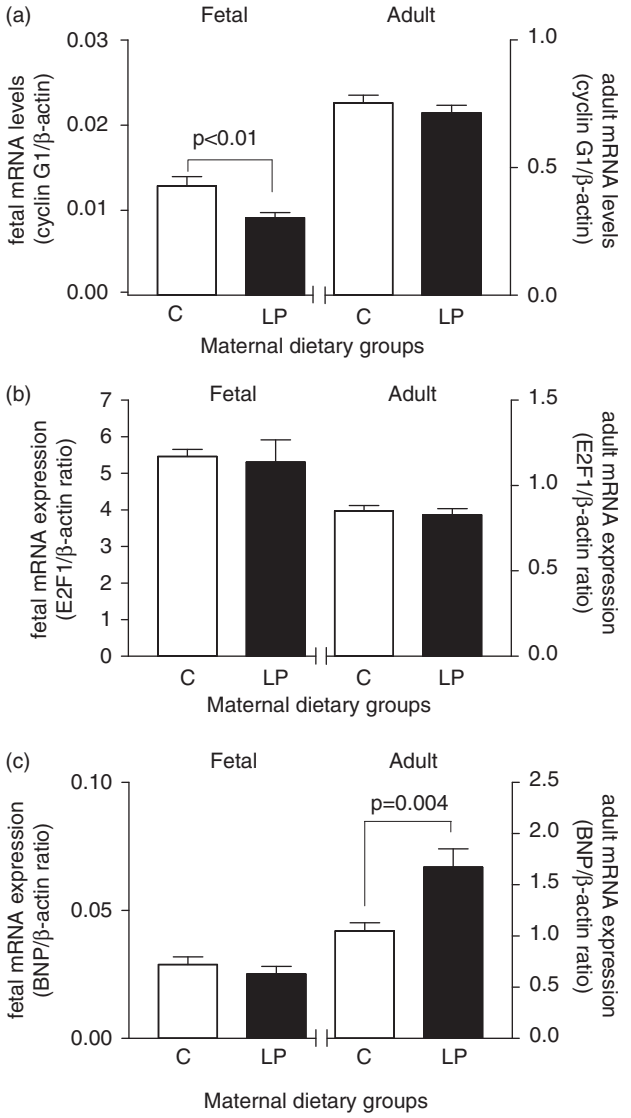

Fig. 1. Cyclin G1 (*a*), E2F1 (*b*) and brain natriuretic peptide (BNP) (*c*) mRNA expression relative to β-actin in the fetal hearts and left venticle of adult male . Values are means ± S.E.M. Statistical differences are indicated by the *P*-value when compared with offspring from C-fed dams. C, standard chow diet; LP, low-protein diet.

origins of CVD. The lower expression of the cyclin G1 gene in the LP fetal heart suggests that cardiomyocyte proliferation is reduced following *in utero* exposure to the LP environment, resulting in the reduction of fetal heart growth and heart volume. This notion is substantiated by a study that has shown that the growth of cardiomyocytes can be inhibited when treated with cyclin-dependent kinase inhibitors.[8] This reduction in fetal heart volume might be an adaptive response of the fetus to survive in a poor nutritional environment. We, however, did not observe significant differences in E2F1 gene expression, which is involved in driving the cell cycle from the G to the S phase during mitosis. This suggests that mitotic activity is not affected by the *in utero* LP environment, and that only certain cell cycle regulators are associated with the inhibition of cardiac growth. It is possible that the activity of RNA polymerase I, II and III and the upstream binding factor (UBF), which regulates RNA synthesis and contributes to the regulation of protein synthesis, may be influenced by the *in utero* LP environment.[18,19] Future studies are needed to assess the activities of UBF and the RNA polymerases in the heart of these LP fetuses.

We saw higher expression levels of E2F1 in fetal *v.* adult hearts because the developmental period is a time of considerable mitotic activity. Interestingly, E2F1 and G1 cyclins, such as cyclin D1 and cyclin G1, are currently being used clinically to reprogram the cardiomyocyte cell cycle to induce cardiomyocyte proliferation for cardiac repair.[20] Thus, knowledge of early cardiomyocyte development could be important for tailoring subsequent treatment to CVD. Even though we did not observe differences in E2F1 in LP compared to the C groups, epigenetic effects on target genes can influence E2F1 activity.[21]

Prenatal exposure to maternal LP diet resulted in the adult offspring being hypertensive and having increased cardiomyocyte size. The increased heart volume and cardiomyocytes in the LP offspring might be a compensatory mechanism for increased blood pressure or disturbed function. In these LP offspring, no differences in the number of Ki 67-positive cells were shown, suggesting that this was not due to late hyperplastic changes. However, there was a higher expression of the BNP gene in the heart tissue of LP adult offspring compared to C group animals, suggesting that their hearts are under greater stress in adulthood, partly from the elevated arterial pressure, and are undergoing tissue remodeling and hypertrophy. These changes in gene expression are in line with our MRI finding of increased heart volume in the LP offspring. It is worth noting that the stress generated by the tail-cuff technique[22] may have contributed to the hypertension in the LP offspring. To reduce the effect of restraint stress on the blood pressure readings, all animals in our study were accustomed to the procedure for 7 days before the actual blood pressure measurement session.

Our observation of lower body weight at birth, followed by no difference in body weight in adulthood, in the LP offspring *v.* the C group indicates that the LP animals exhibited catch-up growth. In rats, reducing such postnatal catch-up growth reduces oxidative stress,[23] and this is known to play a role in the development of cardiac hypertrophy[24] and also in hypertension.[25] Thus, the catch-up growth seen in the LP offspring could result in increased oxidative stress, which may then contribute to the increased heart volume and hypertension seen in these LP animals.

In summary, fetal adaptive responses to unbalanced nutrition can predispose to CVD in adulthood. A response that may be appropriate during fetal development can lead to risk of disease later in life, especially when associated with a more plentiful diet and elevated blood pressure. The increased adult heart volume noted in our mouse study

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resembles human pathology in terms of increased cardiomyocyte size and raised BNP expression. Our present findings warrant further investigation into the effects of diet on the cell cycle regulators and related molecular changes during critical periods of development. This may lead to improved early diagnosis of increased risk of CVD and to novel therapeutic interventions to reduce the risk following unbalanced nutrition in early life.

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Statement of Interest

None

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