Maternal low protein diet programs cardiac beta-adrenergic response and signalling in 3 month old male offspring

Short title: beta-adrenergic response in low protein rat offspring

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Abstract

Low birth weight in humans is associated with an increased risk of cardiovascular disease. Humans with heart failure have a reduced beta-adrenergic response. The aim of this study was to investigate the hemodynamic response to the beta-adrenergic agonist isoproterenol and to identify molecular deficiencies that may be predictive of cardiac failure in a low birth weight rodent model which develops insulin resistance and type-2 diabetes in adulthood. Wistar rats were fed a control or a low protein diet throughout pregnancy and lactation. The resting heart rate and blood pressure of the 3 month old male offspring of these dams, termed “control” and “Low Protein” (LP) groups respectively, and their responses to isoproterenol (ISO) infusion were monitored by radiotelemetry. The protein expression of beta-adrenergic signalling components was also measured by western blotting. Basal heart rate was increased in LP offspring (p<0.04), although mean arterial pressure was comparable with controls. Chronotropic effects of ISO were blunted in LP offspring with significant delays to maximal response (p=0.01), a shorter duration of response (p=0.03) and a delayed return to baseline (p=0.01) at the lower dose (0.1 μg/kg/min). At the higher dose (1.0 μg/kg/min ISO), inotropic response was blunted (p=0.03) but quicker (p=0.001). In heart tissue of LP offspring, β1-adrenergic receptor expression (β1-AR) was reduced (p<0.03). β1-adrenergic receptor kinase (β1-ARK) and both stimulatory (Gsα) and inhibitory G-protein (Giα) levels remained unchanged, while β-arrestin levels were higher (p<0.03). Finally, insulin receptor-β expression was reduced in LP offspring (p<0.012). Low protein offspring have reduced beta-adrenergic responsiveness and attenuated adrenergic and insulin signalling, suggesting that intrauterine undernutrition alters heart failure risk.

Keywords: beta-adrenergic response, beta-adrenergic receptor, insulin receptor, beta-arrestin
INTRODUCTION

Low birth weight in humans is associated with a range of adult diseases including type-2 diabetes, abnormal lipid metabolism and hypertension (4), and increased risk of death from cardiovascular disease (5, 24, 28) and ischemic heart disease in the adult (19, 22, 39, 55). It has been suggested that this may be a consequence of fetal under-nutrition during gestation (4), which induces physiological and/or metabolic adaptations to ensure nutrient supply to vital organs (such as the brain) at the expense of other organs (such as the pancreas) (26). Various other adverse fetal environments have been shown to associate with low birth weight and increased risk of similar diseases. These include fetal hypoxia (3), maternal anemia (53), maternal smoking (25, 46), maternal periodontal disease (44), placental villous inflammation (6) and maternal asthma (12).

The underlying mechanisms of these human observations are poorly understood, however, several experimental animal models of maternal dietary manipulation have provided insight into the causal links between poor fetal growth and subsequent disease (43). The low protein model is one of the most extensively studied of these and has been used to identify key molecular pathways involved in the development of insulin resistance and type-2 diabetes (47). Other studies in this model showed that these low protein male offspring subsequently develop insulin resistance and diabetes in old age (50). Previous studies in this lab showed that male offspring of low protein fed rat dams (LP offspring) demonstrate raised epinephrine and norepinephrine concentrations in the fed state at 12 weeks of age (51). After an overnight fast however, these parameters were increased in the control group but not in LP offspring. In addition, Alpha 2A adrenergic receptor levels in adipocytes isolated from epididymal, subcutaneous
and intra-abdominal fat stores were lower in LP offspring while on the contrary their beta-1 and beta-3 adrenoreceptors were higher than controls.

Prenatal hypoxia in rats also results in low birth weight (32, 41) and has been shown to increase the susceptibility of the adult to ischemia-reperfusion injury (41). The potential mechanisms suggested included increased beta (2)-adrenoreceptor and the G(s)alpha/G(i)alpha ratio, and a decrease in heat shock protein 70 and endothelial nitric oxide synthase in the left ventricle (41). In another study, prenatal hypoxia induced increases in ventricular weights and impaired cardiopulmonary vasoconstriction (32).

These observations led us to hypothesize that intrauterine growth restriction might program long-term alterations in catecholamine sensitivity in the adult, with potential adverse effects on cardiac function. The aim of this study was therefore to investigate the possible causes of raised catecholamines in LP male rats and the potential effects this might have on their cardiovascular system.

In light of the data from the fetal hypoxia model, we also investigated the expression of cardiac β1, β2 and β3 adrenergic receptors as well as downstream signalling components of the beta-adrenergic signalling pathway, i.e. adenylate cyclase (AC) IV, V and VI, β adrenergic receptor kinase-1/G-protein coupled receptor kinase 2 (βARK-1), Gia (inhibitory G protein) and beta-arrestin, to determine whether these mechanisms were involved with in vivo cardiac βAR function.
METHODS

All biochemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated. Female Wistar rats were bred locally at a designated animal unit of the University of Cambridge, UK. Adult females weighing between 235-250g were mated and assumed to be pregnant when a vaginal plug was expelled. They were then fed *ad libitum* either a control diet (containing 20% [w/v] protein) or an isocaloric low-protein (8 % protein) diet (Hope Farms BV, Woerden, Netherlands) during gestation and lactation (See Table A for composition of the diets). Two days after birth, litter sizes were randomly standardised to 4 males and 4 females. At 21 days of age the male offspring were weaned onto a standard rat diet (LAD1; Special Diet Services, Witham, UK) and remained on the LAD1 diet for the remainder of the study. 1 male each from 8 control and 8 LP litters were included in this study. All animal procedures were approved by the Local Animal Ethical Review Committee and were carried out under compliance with the United Kingdom Animal (Scientific Procedures) Act 1986. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

*Surgery*

3 month-old male rats were anaesthetised with halothane (Fluothane; Zeneca, Macclesfield, UK) (4% halothane in oxygen for inducing and 2% for maintaining anaesthesia). Sterile catheters (Esco Rubber, 0.5 mm bore, Bibby Sterilin Ltd., Stone, UK) were placed bilaterally into the jugular veins. The distal ends of the catheters were tunnelled subcutaneously and exteriorised at the nape of the neck. Each catheter was back-filled with heparinised saline (20U/ml) and then plugged. To maintain patency, the heparin block was first aspirated off, the line flushed with
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saline and then the heparin block reinstalled daily. A radiotelemetry device was then implanted for the measurement of mean arterial pressure. The probe was inserted into the descending aorta via a small incision in the femoral artery and the probe and catheter secured with sutures and tissue adhesive. Correct placement of the probe within the aorta was verified by the reception of an intermittent signal corresponding to the animal’s pulse rate when the device was switched on with a magnet. The body of the transmitter was then placed in the peritoneal cavity and secured with sutures into the body wall. Finally, when hemostasis was ensured, the 2 skin incisions were closed with interrupted stitches. The animals received post-operative analgesia (buprenorphine) for a week and were allowed to recover until they appeared to have normal feeding, drinking and grooming behaviour. Stimulation with the beta-adrenergic agonist isoproterenol was performed between 10-14 days after surgery between 11:00 and 14:00 hours for all rats.

Rats remained in their individual cages without restraint throughout the duration of the infusion process and only one rat was infused at a time. After moving to a quiet room, the infusion lines were attached and the rat then placed onto a platform receiver and allowed to acclimatize to the environment for an hour. From this point onwards, heart rate and blood pressures, both systolic and diastolic were sampled continuously. Baseline measurements were established during this quiet time. Saline was then infused for 30 minutes to get the animal used to the infusion process, by the end of which, heart rate and blood pressure fluctuations had settled. Isoproterenol in saline was then administered initially at a dose of 0.1ug/kg/min for 10 minutes via the jugular catheter using a syringe infusion pump (Razel Model A-99) with an adjustable flow rate and 1.0ug/kg/min for 10 minutes via the jugular catheter. This was followed by an infusion of saline at the same rate for 30 minutes before the next higher dose was applied also for 10 minutes. This was followed by a final saline flush for 30 minutes. Recording was then
continued for 1 hour afterwards before removal of infusion lines and return to normal housing. After the experiment, the rats allowed to recover for a week and then killed by decapitation and trunk blood and tissue collected for further analysis.

Telemetry and Data Acquisition

The radiotelemetry system and software used in this study to measure mean arterial pressure and heart rate was obtained from Data Sciences International, St Paul, Minnesota, USA. It is comprised of the implantable transmitter (TA11PA-C40); a receiver (RPC-1) on which the animal, which remains in its own cage throughout, is placed; the multiplexer which consolidates the signals received, and a computer loaded with the software for acquisition and analysis of the data received (Dataquest ART 2.2). For each animal, individual and direct numeric outputs of 10 second intervals were obtained for the parameters of heart rate, mean arterial pressure and systolic and diastolic pressures. From these outputs, various parameters including “maximal change in heart rate or mean arterial pressure”, “delay to maximal response” and “duration of maximal response” subsequent to each dose of isoproterenol were calculated. “Delay to basal heart rate or mean arterial pressure” was calculated as the time taken by each individual animal to return to its own basal pressure after each dose of isoproterenol treatment was removed.

Western blotting

Whole heart lysates were prepared from frozen tissue and protein content determined as described previously (21). Cleared protein lysates were standardised to a final concentration of 2 mg/ml in Laemmli’s sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue and 150 mM DTT). For preparation of cardiac membranes, frozen heart tissue
was ground to a powder in a mortar on dry ice and then mixed with fresh ice-cold buffer containing 5 mM Tris pH 7.4, 2 mM EDTA and protease inhibitors (21). Samples were homogenised with 20 passes before centrifugation at 1000 g at 4°C for 15 minutes to pellet nuclear material. The supernatants were then centrifuged for 1 hour at 100,000 g to pellet cardiac membranes which were subsequently resuspended in lysis buffer (21) and the protein samples standardised to 2 mg/ml in Laemmli’s sample buffer. Samples were boiled for 5 min and then separated by SDS-PAGE. Proteins were transferred onto PVDF membrane (Immobilon-P, Millipore) and Western blotting carried out as previously described (48). Cardiac membranes were probed with antibodies to Adenylate cyclase IV (Santa Cruz sc-20763, a rabbit polyclonal raised against a recombinant protein corresponding to amino acids 631-800 mapping at the carboxy terminus of human adenylyl cyclase IV and cross-reactive with rat and mouse adenylyl cyclase IV); Adenylate cyclase V&VI (Santa Cruz sc-590, a rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of human adenylyl cyclase V, which is identical to the carboxy terminus sequence of rat adenylyl cyclase VI, and cross-reacts with rat and mouse adenylyl cyclases V and VI); inhibitory G-protein (G\textsubscript{ia}; Upstate, Lake Placid, NY #05-465, a rabbit polyclonal raised against a 10 residue synthetic peptide corresponding to the C-terminal region shared by G\textsubscript{ia}-1 and G\textsubscript{ia}-2 and recognizes both rat and mouse G\textsubscript{ia}-1 and 2) and stimulatory G-protein (G\textsubscript{as}: an affinity purified rabbit polyclonal antibody raised against a peptide mapping within the N-terminus of G\textsubscript{as} of human origin, which cross reacts with mouse, rat and bovine G\textsubscript{as}). Whole lysates were probed with antibodies to \(\beta\)1-adrenergic receptor (Affinity Bioreagents, Colorado, a rabbit polyclonal raised against a synthetic peptide mapping to residues 394-408 of mouse, which is identical to the rat sequence and detects \(\beta\)1AR from mouse and rat tissues ); \(\beta\)2-adrenergic receptor (Abcam, Cambridge, UK, a rabbit polyclonal
antibody raised against a synthetic peptide corresponding to the carboxy terminus of human β2AR; β3-adrenergic receptor (Alpha Diagnostics International, Texas a rabbit polyclonal raised against a 20 amino acid mouse β3AR peptide which is 85% homologous to the rat sequence and recognizes mouse and rat β3AR); β-arrestin (Applied Bioreagents PA1-730, a rabbit polyclonal raised against residues 384-397 of human β-arrestin 2 and cross-reactive with rat β-arrestin and β-arrestin 2); Insulin receptor-β subunit (IR-β) (Santa Cruz sc-711; Autogen Bioclear, UK, a rabbit polyclonal raised against the C-terminus of IR-β of human origin and cross-reactive with mouse and rat IR-β). A mouse monoclonal antibody to beta actin with cross-reactivity to rat (Abcam, Cambridge, UK ab-6276) was used as a loading control. Horseradish peroxidase conjugated secondary antibodies to mouse and rabbit were obtained from Amersham, APBiotech, UK. Antibody binding was detected using the ECL kit from Amersham, UK. Optical density of immunodetected bands were measured using AlphaEase gs 3.3b. Western blots were image analysed and the immuno-positive bands measured by spot densitometry. The arbitrary value obtained (Integrated Density Value or IDV) was then subtracted from that of the background of to arrive at corrected IDVs. The corrected IDVs obtained from both the control and LP samples were then normalised to actin, detected by an actin antibody on either the lower half of the same blot or the same blot stripped and re-probed with actin antibody. Inter and intra-group c.v. of actin levels were analysed and found to be within 5%. These normalised values then undergo statistical tests. Control means are then set at 100% ± sem and then the LP values are calculated as a percentage of the controls.
Adrenal gland morphometric measurements

Adrenal glands from control and LP groups were removed at post-mortem and fixed in 4% formalin for 16 hours before moving to 70% ethanol and then processed to wax. 4 μm sections were cut to include the largest cut surface along the longest plane and stained with hemotoxylin and eosin. Medullary areas from each section were quantified with the image analysis program analySIS (Olympus, UK) and the largest measurement from each animal was included for statistical analysis.

Plasma analysis

Animals were killed by CO₂ asphyxiation between 9 and 11 in the morning. ACTH and corticosterone levels of these animals (see results) were comparable to the least stressful method of killing as described in Vahl et al, 2005 (57). Fasting blood was collected by decapitation and EDTA plasma was stored at -80°C until used. Plasma insulin concentrations were measured using a Rat Insulin Elisa kit (Mercodia Ultra-sensitive Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden). Plasma corticosterone and Adrenocorticotropic hormone were measured with ELISA kits from Immunodiagnostic Systems Ltd (IDS, Tyne & Wear, UK). All samples were assayed in duplicate and an intra-assay coefficient of variation of up to 5% was accepted.

Statistical Analysis

Data are presented as means with standard error of the mean (S.E.M.) and comparisons between groups were assessed by unpaired two-tailed t-tests using GraphPad Instat (Statistical Solutions, UK) unless stated otherwise. p values of <0.05 were considered statistically significant.
RESULTS

*In-vivo hemodynamic measurements*

The basal heart rate of the LP group was increased compared to controls (Table 1). The increases in heart rate following isoproterenol infusion were comparable in LP and control rats for each of the doses of 0.1 and 1.0 μg/kg/min (Table 1). There was however a significant delay before maximal response in the LP group compared to controls, with controls taking less time to reach maximal response than LPs at the lower dose (Figure 1A). The duration of maximal response was also reduced for LPs at this dose. In addition, the recovery time to basal heart rate was significantly extended in LPs. With the higher dose, the LP group reached maximal stimulation quicker, although the amplitude of the response (Table 1) and the duration of the response at this dose were not different between the 2 groups (Figure 1A).

Basal mean arterial pressures, as well as diastolic and systolic pressures were comparable between the 2 groups (Table 2). The attenuation in mean arterial pressure was greater at the higher dose of isoproterenol, however the magnitude of the responses were similar between the 2 groups (Table 2). The response times were also similar for the lower dose (Figure 1B). At the higher dose however, the LPs achieved maximal response in significantly less time than the controls (Figure 1B) although the duration of their response was reduced.

*Beta-adrenergic receptor expression*

β1-AR expression in LP hearts was reduced compared to controls (p=0.038; Figure 2a). β2 and β3 AR expression were however, comparable in both groups (Figure 2b and 2c). There were no differences in the protein levels of β1 and β3-AR in aorta of controls and LP rats (not shown). β2 expression was undetectable in this tissue.
Expression of downstream signalling molecules

The expression of adenylate cyclase IV and V/VI protein in LP heart membranes were found to be comparable to controls (Fig 3a & 3b). Levels of both $G_{s\alpha}$ and $G_{i\alpha}$ were also comparable (Fig 3c and d respectively). $\beta_1$ARK levels were also unaltered in the LP group (Fig 3e; p=0.57). $\beta$-arrestin levels were however raised by more than three-fold in LP whole heart lysates (Fig 3f; p=0.032).

Insulin signalling

The protein levels of Insulin receptor $\beta$-subunit were reduced by half in the LP heart tissue compared to the control group (Fig. 4; p= 0.013).

Body weights and morphometry of adrenal glands

Birth weights, weights at weaning and at the time of the experiment were found to be significantly lower in the LP group (p<0.001 at birth and at weaning and p<0.05 at 3 months of age; Table 3). The adrenals weights from both groups were comparable (Table 3), however adrenal to body weight ratio of the LP group was significantly higher than the control group (p=0.0167; Table 3). LP medullary area was also higher than controls (p=0.005; Table 3).

Plasma Data

Plasma glucose in low protein offspring was lower than that of controls. Insulin levels also tended to be reduced in the LP group. Adrenocorticotropic hormone (ACTH) levels were comparable between the 2 groups, as were corticosterone levels (Table 4). ACTH and
corticosterone levels in both groups are comparable to the least stressful method of killing and exsanguinations.
DISCUSSION

Epidemiological studies have shown low birth weight to be related to the development of age-related diseases including type-2 diabetes (27), hypertension, cardiovascular disease (17, 23) and obesity (60). The rat low protein model is well documented in its applicability to the study of metabolic disease pathologies. It results in low birth weight offspring and the development of insulin resistance and frank diabetes in older male offspring (50) and an insulin resistant state in female offspring (21). Nutrition during the suckling period also has a huge developmental impact as shown by the epidemiological observations of Eriksson et al (2001) (18), which suggest that low weight gain during the first year of infancy is also crucial in the development of coronary heart disease. In this model, the LP mother’s diet is maintained at 8% protein during lactation also, which reduced weight gain during this period.

The present study has identified that the increased peripheral epinephrine levels reported previously for low protein male offspring rats (51) is likely due to an increased adrenal:body weight ratio and an increase in medullary area. We also found their resting heart rate to be raised which is consistent with the raised epinephrine.

The overall delay in the response as well as the reduced duration and magnitude of response implies a reduced cardiac and arterial β-adrenergic responsiveness. Upon activation by the lowest dose, the low protein offspring were unable to sustain their maximal response and heart rate started to decline even before the end of the infusion. Interestingly however, they took longer to return to the basal heart rate. During the higher dose however, low protein offspring responded more quickly, but less strongly compared to the controls. This led us to investigate if components of beta adrenergic signalling were altered in LP heart tissue.
β-adrenergic receptors belong to the larger family of G-protein-coupled receptors (GPCRs) which modulate cardiac function by controlling the inotropic and chronotropic response to catecholamines. β-1AR is coupled to stimulatory G protein (Gs) and its phosphorylation by the β1AR kinase (β1ARK) leads to a blockade of downstream-signalling and desensitization of the receptor to further catecholamine stimuli. Chronic over-stimulation of the cardiac β-adrenergic system is toxic to the heart and may contribute to the pathogenesis of congestive heart failure (52). Numerous studies have shown a decrease of the cardiac β-adrenergic receptors in failing hearts (8-10, 16, 34), specifically a reduction of the β1 subtype protein levels and up to 50% reduction in its mRNA, which correlated to disease severity. An accompanying increase in Giα (15), has been shown to reduce the responsiveness of Gs-coupled receptor systems such as the β1 adrenergic receptor in diseased human myocardium (7) and in over-expression systems (14, 31, 54). We observed that the β1 adrenergic receptor, which is the predominant cardiac subtype and provides the strongest stimulus for cardiac function (35), was reduced in the LPs. This is consistent with their reduced responsiveness to isoproterenol. We propose that the increased basal epinephrine previously observed for LP male offspring of a similar age (51) is an adaptive response to decreased cardiac β1AR expression observed here, as a means of maintaining cardiac function. Experimental agonist stimulation coupled to the reduced number of β1-adrenergic receptors in the heart led to a progressive desensitization of the available receptors as evidence by the shortened duration of activity and prolonged delay to basal heart rate and pressure after removal of the stimulus. β1ARK expression was not altered in LPs, which mirrors the observations of Leineweber et al, 2003, for the aging heart (38), and neither was Giα expression. However, LP β-arrestin protein levels were raised which agrees with observations of reduced beta adrenergic signalling also in aging (13) as well as failing hearts.
(58). This suggests that the hearts of LPs might be ageing more quickly. Recent studies have implicated a role for β-arrestin in the regulation of beta adrenergic receptor desensitization after agonist binding (2, 49), by acting to slow the rate of cAMP production and increasing the rate of its degradation at the membrane by its association with phosphodiesterase enzymes (1).

Unlike some low protein diets, the one used in the current study did not affect blood pressure, which is consistent with the findings of others using the same diet (36). This may be due to some differences in diet compositions.

This study provides evidence showing that maternal under-nutrition programmes adrenal growth and adrenomedullary hormone secretion. One possibility is that this may be a direct response to the young low protein offspring’s mild hypoglycemic state, which would cause levels of epinephrine, a short term glucose counterregulatory hormone to rise (33). Persistent adrenergic stimulation might then lead to the observed down-regulation of β1-adrenergic receptor levels in the heart, as an adaptive response, which could be protective against heart failure as this is effectively a beta block response. On the other hand, the increased level of β-arrestin is consistent with a requirement for rapid desensitization of the reduced receptor numbers to allow rapid recycling to the plasma membrane. The overall likely effect is one of reduced initial response due to the reduced receptor density, but a more rapid response at a higher stimulatory dose, as more arrestin would allow faster uncoupling of the receptor from Gs and return of the available receptors to the plasma membrane. This adaptive response is beneficial in young animals, however, with age, LPs develop a worsening of their glucose tolerance (50) leading to hyperinsulinemia. It has been shown that cross-talk between beta adrenergic and insulin receptors in neonatal rat cardiomyocytes exists and that beta-adrenergic receptor stimulation has a biphasic effect on insulin-stimulated glucose uptake. While short-term
stimulation induces an additive effect on insulin-induced glucose uptake, long-term stimulation inhibits both insulin-stimulated glucose uptake and insulin-induced autophosphorylation of the insulin receptor (45). Thus the increased peripheral epinephrine and downregulation of insulin receptor density in the LPs suggests they may develop cardiac insulin resistance with age. This is supported by human studies which demonstrate a role for diminished insulin signalling and insulin resistance in the development of cardiomyopathy (29) and heart failure (20) with age.

Various other animal models used to study the effects of human fetal growth restriction support the link between intrauterine growth restriction and cardiovascular disease. In sheep (59), foetuses of ewes nutrient restricted during days 28-78 of gestation showed compensatory left ventricular growth which was associated with increased transcription of genes related to cardiac hypertrophy, compensatory growth or remodelling (30). In rats exposed to chronic hypoxia during fetal development, the cross sectional area of a left ventricular (LV) myocyte was increased and response to heat stress was inhibited (40). Most recently, rat offspring of a low protein pregnancy were shown to have reduced heart weight and cardiomyocyte number (11).

Children born small for gestational age have been shown to have increased epinephrine levels in circulation (56). Low birth weight is also associated with coronary heart disease in adults and with vascular endothelial dysfunction in children (37, 42). Crucially, they also develop insulin resistance earlier in life (27). This study provides insight into possible mechanisms by which low birth weight and low weight gain during infancy may impact on subsequent heart failure risk.
ACKNOWLEDGEMENTS

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Figure legends

Figure 1: Timing of heart rate and mean arterial pressure responses to isoproterenol

Responses to two doses of isoproterenol, 0.1 μg/kg/min (ISO 1) and 1.0 μg/kg/min (ISO 2) in 3 month old Control and Low Protein ‘LP’ rats were timed and the delay to maximal response, duration of the maximal response and delay to basal values plotted for (A) Heart rate responses and (B) Mean arterial pressure responses. Control values are represented in white bars and LP values in shaded bars. Data are presented as means ± S.E.M.; n=8 for both groups; * p<0.05; ** p<0.01.
**Figure 2: beta adrenergic receptor expression in heart**

The expression of (a) β1-adrenergic receptor; (b) β2-adrenergic receptor and (c) β3-adrenergic receptor in whole heart lysates of 3 month-old male control and LP rats was determined by Western blotting of heart samples taken from Control ‘CONTROL’ and Low Protein ‘LP’ offspring rats as described in Methods. White bars, control groups; hatched bars, LP groups. Results are expressed as means ± S.E.M. *p<0.05.*
Figure 3: Expression of beta adrenergic downstream signalling molecules in heart

The expression of (a) Adenylate cyclase IV; (b) Adenylate cyclase V & VI; (c) inhibitory G protein, Giα; (d) β1-adrenergic receptor kinase and (e) β -arrestin in cardiac membranes (a, b & c) and whole heart lysates (d & e) of 3 month-old male control and LP rats was determined by Western blotting of heart samples taken from Control ‘CONTROL’ and Low Protein ‘LP’ offspring rats as described in Methods. White bars, control groups; hatched bars, LP groups. Results are expressed as means ± S.E.M. *p<0.05.
Figure 4: Expression of insulin receptor in heart

The expression of insulin receptor-β subunit in whole heart lysates of 3 month-old male control and LP rats was determined by Western blotting of heart samples taken from Control ‘CONTROL’ and Low Protein ‘LP’ offspring rats as described in Methods. White bars, control groups; hatched bars, LP groups. Results are expressed as means ± S.E.M. *p<0.05.
Table A: Composition of gestational and lactational diets shown as percentage by dry weight of constituents

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Total: 100.00 100.00
Table 1. Magnitude of heart rate responses to isoproterenol infusion

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Iso 1 – Isoproterenol at 0.1 μg/kg/min; Iso 2 – Isoproterenol at 1.0 μg/kg/min. Values are mean with s.e.m. in parentheses. HR- heart rate; b.p.m- beats per minute

# Mann-Whitney non-parametric tests were applied due to the dissimilar variances.
Table 2. Magnitude of mean arterial pressure responses to isoproterenol infusion

<table>
<thead>
<tr>
<th></th>
<th>Control n=8</th>
<th>LP n=8</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure (MAP) (mmHg)</td>
<td>111.2 (3.1)</td>
<td>115.7 (5.3)</td>
<td>0.53</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>99.1 (7.1)</td>
<td>98.1 (3.9)</td>
<td>0.90</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>133.4 (4.7)</td>
<td>132.5 (3.9)</td>
<td>0.90</td>
</tr>
<tr>
<td>Iso 1 Max. change in MAP (mmHg)</td>
<td>12.8 (2.0)</td>
<td>15.2 (1.9)</td>
<td>0.46</td>
</tr>
<tr>
<td>diastolic pressure (mmHg)#</td>
<td>14.2 [12.5-15.7]</td>
<td>15.0 [11.8-18.6]</td>
<td>0.80</td>
</tr>
<tr>
<td>systolic pressure (mmHg)#</td>
<td>15.2 [12.1-16.5]</td>
<td>14.8 [11.4-17.9]</td>
<td>0.94</td>
</tr>
<tr>
<td>Iso 2 Max. change in MAP (mmHg)</td>
<td>26.8 (1.4)</td>
<td>26 (7.8)</td>
<td>0.84</td>
</tr>
<tr>
<td>diastolic pressure (mmHg)#</td>
<td>25.5 [23.5-27.6]</td>
<td>24.7 [19.9-33.3]</td>
<td>0.94</td>
</tr>
<tr>
<td>systolic pressure (mmHg)#</td>
<td>25.9 [22.3-27.2]</td>
<td>25.6 [18.5-37.5]</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Iso 1 – 0.1 μg/kg/min; Iso 2 – 1.0 μg/kg/min. MAP- mean arterial pressure; mmHg- millimeters of mercury. Values are means with s.e.m. in parentheses except for # which were analyzed by non-parametric tests and values presented as medians with interquartile ranges in square brackets.
Table 3. Anatomical parameters

<table>
<thead>
<tr>
<th></th>
<th>Control n=8</th>
<th>Low protein n=8</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birthweight (g)</td>
<td>6.56 (0.19)</td>
<td>5.55 (0.09)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Body weight at weaning (g)</td>
<td>49.4 (1.11)</td>
<td>29.74 (1.07)</td>
<td>1.96e-10</td>
</tr>
<tr>
<td>Body weight at 3 months (g)</td>
<td>416 (12)</td>
<td>376 (12)</td>
<td>0.029</td>
</tr>
<tr>
<td>Adrenal weight (mg)</td>
<td>73 (6)</td>
<td>80 (4)</td>
<td>0.4</td>
</tr>
<tr>
<td>Adrenal weight: Body weight</td>
<td>0.00018 (1.2E-05)</td>
<td>0.00022 (9.2E-06)</td>
<td>0.017</td>
</tr>
<tr>
<td>Medullary area (μm²)</td>
<td>1.58E+06 (2.5E+04)</td>
<td>2.12E+06 (1.33E+04)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*Values are means +/- s.e.m.*
Table 4: Plasma analysis

<table>
<thead>
<tr>
<th></th>
<th>Control n=8</th>
<th>Low protein n=8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.2 (0.1)</td>
<td>4.8 (0.1)$^5$</td>
</tr>
<tr>
<td>Insulin# (pmol/L)</td>
<td>192 [94-209]</td>
<td>119 [15-157]</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>14.6 (3.1)</td>
<td>12.6 (2.2)</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>88.3 (23.6)</td>
<td>78.8 (14.1)</td>
</tr>
</tbody>
</table>

Values are means +/- s.e.m. for all measurements except for insulin# which were analyzed by non-parametric tests and values presented as medians with interquartile ranges in square brackets. $^5 p=0.058.$
References


27. **Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, and Winter PD.** Fetal and infant growth and impaired glucose tolerance at age 64. *Bmj* 303: 1019-1022, 1991.


Figure 1

A  Heart rate response to isoproterenol

B  MAP response to isoproterenol
Figure 2

(a) $\beta_1$-AR

(b) $\beta_2$-AR

(c) $\beta_3$-AR
Figure 3

a. AC-IV

b. AC-V & VI

c. Gsα

d. Giα

e. β1ARK

f. β-arrestin
Figure 4

IR-β

Control LP

% of Control Means

Control LP

0.0 20.0 40.0 60.0 80.0 100.0 120.0

*