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Sex differences in sensitivity to β-adrenergic agonist isoproterenol in the isolated adult rat heart following prenatal protein restriction

Matthew J. Elmes[1]*, Andrea Haase[1], David S. Gardner[2] and Simon C. Langley-Evans[1]

[1]*Division of Nutritional Sciences, University of Nottingham, Loughborough, Leicestershire LE12 5RD, UK*
[2]*School of Veterinary Medicine and Science, University of Nottingham, Loughborough, Leicestershire LE12 5RD, UK*

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Hypertension is a major risk factor for the development of CVD. Epidemiological studies have shown that low birth weight increases the risk of developing hypertension in adulthood. Hypertension increases the risk of suffering IHD and early findings provide evidence that hearts from prenatally protein-restricted, hypertensive, male offspring are more susceptible to cardiac dysfunction following ischaemic events. Hypertension and abnormalities in cardiac function following ischaemia–reperfusion in the human population are treated therapeutically with β-adrenergic antagonists. We hypothesised that increased susceptibility to myocardial ischaemia–reperfusion injury in prenatally programmed offspring may be due to sympathetic hyperactivity. Pregnant Wistar rats were fed control or low-protein (maternal low protein; MLP) diets throughout gestation. At age 6 months, hearts were rapidly excised and retro-perfused using the Langendorff apparatus, to assess isolated cardiac function following stimulation with increasing doses of the non-specific β-agonist isoproterenol. Baseline heart rates were similar in control and MLP-fed offspring. With significant diet × sex interactions ($P<0{\cdot}01$) maximum heart rate response following isoproterenol infusion was significantly longer in MLP than control. Prenatal diet had no effect on maximal left ventricular developed pressure (LVDP) response, but the LVDP isoproterenol response was significantly longer in duration in MLP-exposed male offspring (diet × sex $P<0{\cdot}001$). Myocardial mRNA expression of β2-adrenergic receptors was increased in 2-week-old female MLP offspring only ($P<0{\cdot}049$). In conclusion, maternal protein restriction programmes cardiac sympathetic activity in a sex-specific manner, and may explain increased susceptibility to ischaemia–reperfusion injury in males subject to fetal undernutrition.

Protein restriction: Heart: β-Adrenergic agonists: Fetal programming

Hypertension has been identified by the WHO as the second major cause of death in developed countries[1]. In England, 34 % of men and 30 % of women have raised blood pressure and it is estimated that 80 % of these individuals are not receiving treatment. Epidemiological studies have shown a clear relationship between characteristics at birth and the occurrence of CVD in adult life[2–4]. Size at birth is an early indicator of disease risk. In particular, low birth weight[2] or thinness at birth[3] is associated with the development of hypertension. These observations have led to the hypothesis that adult CVD arises as a result of impaired fetal growth and development *in utero*, following suboptimal nutrition during the early developmental stages of life[4]. Elevated blood pressure and cardiovascular dysfunction are commonly reported results of intra-uterine exposure to maternal undernutrition in rodents[5] and sheep[6].

A common therapeutic strategy aimed at decreasing high blood pressure and myocardial ischaemia–reperfusion injury includes the use of β-adrenergic antagonists. This is based on the fact that sympathetic hyperactivity is a causal factor in hypertension. This has prompted us to suggest that the offspring of animals subject to undernutrition in pregnancy may show signs of sympathetic hyperactivity. This may play a key role in the development of hypertension and increased susceptibility to myocardial ischaemia–reperfusion injury. Work in our laboratory has shown that protein restriction in rat pregnancy can programme both of these adverse cardiovascular outcomes[7].

The sympathetic nervous system provides one of the most important mechanisms for the regulation of cardiac function, through β-adrenergic receptor stimulation. The inotropic and chronotropic responsiveness of the heart to β-adrenergic stimulation deteriorates with age[8] and these changes are observed both *in vivo* [9] and *in vitro* [10]. At birth the sympathetic innervation of the heart in most mammals is poorly developed, but the adrenoreceptors are present well before[11]. During postnatal development the contractile apparatus matures[12] and this is associated with profound changes in the modulatory effect of β-adrenoreceptor signalling. Novotny *et al.* [13] suggested that the differential maturation of various components of the signal transduction pathway may contribute to complex age-dependent changes in the cardiac responsiveness and sensitivity to β-adrenergic stimulation.

It is well established that food restriction of the adult rat modifies β-adrenergic responsiveness of the heart[14,15], suggesting that these pathways are responsive to nutritional

Abbreviations: AUC, area under the curve; dP/dt_{max}, rate of pressure development; LVDP, left ventricular developed pressure; MLP, maternal low protein.

* **Corresponding author:** Dr Matthew Elmes, fax +44 115 951 6122, email matthew.elmes@nottingham.ac.uk

modulation. At present, very few studies have investigated the possible programming effects of varying maternal nutrient intake in pregnancy upon maturation of the cardiac β-adrenergic receptors. This is an important omission as this could be a factor in the development of adult hypertension and cardiac dysfunction. The primary hypothesis of the present study was therefore that prenatal exposure to protein restriction programmes the β-adrenergic signalling pathway and, as a result, cardiac function. Thus programming of sensitivity to β-agonists could be an important mechanism through which prenatal protein restriction determines increased susceptibility to ischaemia–reperfusion injury. To test this theory we used isolated heart Langendorff preparations from control and protein-restricted offspring to assess the inotropic and chronotropic responsiveness of the heart to β-adrenergic stimulation, and measured myocardial gene expression of β_1- and β_2-adrenergic receptors.

Materials and methods

Animals

Twelve virgin Wistar rat dams (Harlan Ltd, Belton, Leics, UK) weighing between 250 and 300 g were mated in the animal facilities at the University of Nottingham. When the appearance of a vaginal plug on the cage floor confirmed mating, the rats were allocated to be fed either a control diet (180 g casein/kg; *n* 6) or a low-protein diet (90 g casein/kg; *n* 6), as described previously in detail[16]. The pregnant rats were then fed the isoenergetic semi-synthetic diets throughout gestation until birth at 22 d gestation. All mothers were then transferred to a standard laboratory chow diet (B&K Universal Ltd, Hull, UK) and each litter culled to a maximum of eight pups to minimise variation in the nutrition of the offspring during suckling. At age 4 weeks all offspring were weaned onto the chow diet, to ensure that litters from control and low-protein-fed rat dams only differed in terms of their prenatal nutrition. All experimental procedures were performed in accordance with the UK Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

The isolated heart (Langendorff) preparation

At age 6 months, one male and one female rat from each litter were randomly selected, anaesthetised using 3 % isofluorane in 2 litres O_2/min and killed by cervical dislocation. The heart was then excised and cannulated via the aorta to Langendorff perfusion apparatus within 90 s (AD Instruments, Oxford, Oxon, UK) and perfused with Krebs–Henseleit buffer (118 mM-NaCl, 4·7 mM-KCl, 1·2 mM-KH_2PO_4, 1·2 mM-$MgSO_4$, 25 mM-$NaHCO_3$, 11 mM-glucose and 1·25 mM-$CaCl_2$ (pH 7·4)) bubbled with O_2–CO_2 (95:5, v/v) in a coronary retrograde fashion. Perfusion pressure was maintained at a constant pressure of 60 mmHg, with perfusate warmed to 37·4°C, and the heart immersed in a water-jacketed temperature-controlled glass chamber set at 37·4°C. This ensured normothermia throughout the perfusion protocol. Contractile function was monitored by the careful insertion of a saline-filled latex balloon (Linton Instruments, Diss, Norfolk, UK) into the left ventricle which was adjusted to an end diastolic pressure of between 5 and 10 mmHg. Left ventricular and perfusion pressure were continuously monitored through pre-calibrated physiological pressure transducers (Senso-Nor 844; AD Instruments). Data for heart rate, left ventricular developed pressure (LVDP) and left ventricular first derivative (dP/dt_{max}) (a parameter of global left ventricular function which measures the rate of increased pressure within the left ventricle) were collected and processed using the Powerlab Data Acquisition System (AD Instruments). Data recording was not started until all variables were stable (15–20 min).

Isoproterenol dose response

Following the equilibrium period, each heart from control (six males and six females) and maternal low-protein (MLP; six males and six females)-exposed offspring was used to generate a dose–response curve by administering 100 μl of increasing doses of isoproterenol (1·1–560 nM; Sigma), dissolved in Krebs–Henseleit buffer, by syringe via the heat block junction of the Langendorff apparatus. Following each dose the heart was allowed to completely recover to its baseline function and remain at that level for 1 min before receiving the next dose of isoproterenol.

Myocardial β_1- and β_2-adrenergic receptor mRNA expression

Total RNA was isolated from snap-frozen hearts from 2-week-old control or MLP-fed offspring (*n* 7–11) using the TRIzol method (Invitrogen, Renfrew, Renfrewshire, UK). Isolated RNA was treated with DNase (Promega, Southampton, Hants, UK) and extracted using phenol–chloroform and precipitated with ethanol. Total RNA (0·5 μg) was reverse transcribed using Moloney Murine Leukemia Virus (MMLV) RT (Promega). Real-time PCR was performed using an ABI prism 7700 sequence detection system (Applied Biosystems, Warrington, Cheshire, UK). Template-specific primer pairs and oligonucleotide probes (Sigma-Genosys, Cambridge, Cambs, UK) specific to β_1- and β_2-adrenergic receptors and the housekeeping gene β-actin were designed using Primer Express v1.5 (Applied Biosystems). The full sequences of primers and probes are shown in Table 1. Each primer set was tested under Taqman PCR conditions using rat genomic DNA as a template. A negative template control and relative standard curve were included in the PCR run. Each standard curve was made by pooling a cDNA sample at dilutions of 0·05, 0·1, 0·2, 0·4, 1·0, 2·5 and 5·0. The target mRNA was quantified by calculating from the standard curve and normalising to β-actin expression.

Statistical analysis

All results are presented as mean values with their standard errors with $P \leq 0{\cdot}05$ considered significant. Individual dose–response data points for heart rate, LVDP and dP/dt_{max} were taken for every second recorded. Male and female data were combined and analysed by two-way ANOVA (SPSS version 9.0; SPSS, Inc., Chicago, IL, USA) to assess whether there were any significant effects of sex, diet or an interaction on cardiac responses to isoproterenol. As statistical analysis revealed significant effects of sex, diet and their interaction, all data are presented for males and females separately. For dose–response curves each replicate (*n* 6) was considered as an individual point and not the group mean. Each dose–response curve therefore

Table 1. Primer sequences

Gene	Primer and probe sequence	Genebank accession
β_1-Adrenergic receptor		
Forward	5′ AGA GCG ACG AAG CGC G 3′	
Reverse	5′ ACG AAA TCG CAG CAC TTG G 3′	NM_012701
Probe	5′ CGC TGC TAC AAC CTA C 3′	
β_2-Adrenergic receptor		
Forward	5′ CCG GCC ATG GAG CCA 3′	
Reverse	5′ TCC ATT GGG TGC CAG CA 3′	BC086538
Probe	5′ CGG GAA TGA CAG CGA C3′	
β-Actin		
Forward	5′ TTC AAC ACC CCA GCC ATG T 3′	
Reverse	5′ GTG GTA CGA CCA GAG GCA TAC A 3′	NM_031144
Probe	5′ CGT AGC CAT CCA GGC TGT GTT GTC C 3′	

represents the mean of six curves. The dose–response curves were fitted using the standard least squares (ordinary) fit method. The effects of maternal diet on $\log EC_{50}$ values and the steepness of the curve (Hill slope) for heart rate and LVDP were analysed by GraphPad Prism (version 5; GraphPad, Inc., San Diego, CA, USA) using a sigmoidal dose–response (variable slope) curve to test the null hypothesis that $\log EC_{50}$ and Hill slope were the same for each dataset. The sigmoidal dose–response curve (variable slope) is defined by the four-parameter logistic equation $y = \text{bottom} + (\text{top} - \text{bottom})/1 + 10^{(\log EC_{50} - x)}\,\text{Hill slope}$, where bottom is the y value at the bottom plateau and top is the y value at the top plateau. $\text{Log}\,EC_{50}$ is the x value when the response is halfway between the bottom and top of the curve. Hill slope describes the steepness of the curve. A standard sigmoid dose–response curve has a Hill slope of 1·0, and is considered to be shallow or steep when the slope is less than or greater than 1·0 respectively. The sigmoidal dose–response curve highlights several issues; it defines the minimum, maximum and the magnitude of the response. The Hill slope tells us how rapidly the response moves from the minimum to maximum response. Furthermore the $\log EC_{50}$ gives an indication as to whether the response occurs at a low or high dose. The Hill slope and EC_{50} values shown in the paper represent mean and SE from values calculated for each individual animal (six per group). Comparison of myocardial expression of β_1- and β_2-adrenergic receptors between dietary groups was analysed by two-way ANOVA (SPSS version 9.0; SPSS, Inc.) with sex, diet and their interaction set as fixed effects.

Results

Ex vivo *heart responses to isoproterenol*

The magnitude of cardiac responses to different doses of isoproterenol can be seen in Table 2. This shows three representative doses (out of fourteen administered) that were chosen to illustrate the cardiac response to a low, medium and high concentration of isoproterenol. Basal contraction rates in hearts from animals exposed to a low-protein diet *in utero* were not dissimilar. A significant interaction between sex and diet was observed for the maximum heart rate achieved following isoproterenol infusion at doses of 8·8 and 560 nM ($P<0·001$ and $P=0·02$ respectively). In each case, hearts from male offspring exposed to a MLP diet had a greater response to isoproterenol. In addition the area under the curve (AUC) for heart rate response to isoproterenol was also influenced by the interaction of diet and sex, but this was only significant ($P<0·01$) at the higher dose of 560 nM. The recovery to baseline was consistently longer in MLP male offspring, but in contrast female MLP hearts took a considerably shorter time to return to basal levels when compared with controls (Fig. 1).

Baseline LVDP was not dissimilar between the two groups of males but was clearly higher in female offspring exposed to the MLP diet *in utero* (diet × sex interaction $P=0·04$). The maximal response to LVDP following isoproterenol treatment in hearts from all animals was not influenced by diet, sex or its interaction at any dose. However, the return of LVDP to basal levels following isoproterenol treatment was affected by all factors ($P<0·001$), but only at the low dose of 8·8 nM. Males at this dose exhibited a significant delay to baseline recovery, producing a greater AUC in MLP offspring when compared with controls. In contrast, the female response was similar in the two groups (Fig. 1).

Baseline dP/dt_{max} was shown to be significantly affected by diet, with MLP exposure *in utero* increasing the rate of pressure change within the left ventricles of both male and female hearts when compared with controls (Table 2; $P<0·05$). The peak rate of pressure change was not significantly different at isoproterenol doses of 70 and 560 nM, but at 8·8 nM there was an interaction between sex and diet ($P=0·04$). From Table 2 it is clear that, in females, prenatal undernutrition caused a significant fall in the peak dP/dt_{max}. The return of dP/dt_{max} to baseline levels was significantly longer in the hearts from MLP-fed offspring, at doses of 70 and 560 nM ($P=0·04$ and $P<0·05$ respectively). A similar trend was noted at 8·8 nM-isoproterenol, but this did not achieve statistical significance ($P=0·07$).

Dose–response curves

The effect of different doses of isoproterenol on the chronotropic (heart rate) response of the isolated rat heart can be seen in Fig. 2 and Table 3. MLP-exposed males were more sensitive than controls to β-adrenergic stimulation, as evidenced by the lower $\log EC_{50}$ and steeper Hill slope values ($P=0·002$; Fig. 2 (a) and Table 3). In contrast, these parameters did not differ significantly between females from the two maternal dietary groups (Fig. 2 (b) and Table 3). Comparison of AUC revealed that both $\log EC_{50}$ and curve gradient were not significantly affected by maternal diet in female offspring (Fig. 2 (d) and Table 3). In contrast, when considering

Table 2. Magnitude of cardiac responses to different doses of isoproterenol*
(Mean values with their standard errors)

	Males				Females				*P*		
	Control (*n* 6)		MLP (*n* 6)		Control (*n* 6)		MLP (*n* 6)				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Sex	Diet	Interaction
Baseline HR (beats per min)	290·9	9·4	290·5	19·3	294·6	10·4	321·6	11·1	0·19	0·31	0·30
MAX HR (beats per min)											
8·8 nM	564·0	47·1	714·3	22·5	724·1	89·2	614·3	103·4	0·26	0·19	<0·001
70 nM	717·8	71·9	750·4	35·0	815·1	27·8	700·6	79·2	0·67	0·46	0·19
560 nM	627·9	64·3	789·1	34·0	978·0	58·1	752·4	102·0	0·10	0·90	0·02
AUC HR (beats per min × min)											
8·8 nM	65 658·0	7290·8	85 029·0	7443·3	84 509·0	6409·7	79 266·0	7193·9	0·37	0·33	0·10
70 nM	90 179·0	3164·3	110 055·0	7645·9	107 362·0	10 194·0	84 944·0	5001·8	0·81	0·61	0·13
560 nM	96 307·0	11 885·9	131 810·0	11 895·7	137 437·0	11 967·3	99 007·0	14 068·7	0·74	0·91	0·01
Baseline LVDP (mmHg)	36·4	4·4	32·8	5·4	25·8	3·6	41·9	4·7	0·87	0·18	0·04
MAX % LVDP											
8·8 nM	154·0	22·6	302·4	115·7	250·2	69·6	238·5	84·5	0·84	0·39	0·31
70 nM	337·2	87·4	537·8	134·0	430·9	48·4	411·1	138·8	0·76	0·46	0·22
560 nM	415·9	124·9	457·0	101·2	345·9	76·9	450·8	176·8	0·76	0·55	0·79
AUC % LVDP (% change from baseline mmHg × s)											
8·8 nM	22 363·0	1320·4	34 592·0	7414·3	31 754·0	4019·5	29 716·0	3330·1	0·001	0·001	0·001
70 nM	28 193·0	4008·2	49 962·0	9866·0	37 768·0	2710·0	42 589·0	10 179·6	0·99	0·09	0·17
560 nM	35 350·0	6398·1	47 662·0	7180·1	36 988·0	6420·2	46 028·0	11 025·9	1·00	0·18	0·84
Baseline d*P*/d*t* (mmHg/s)	1416·4	195·8	1638·1	76·4	1360·8	144·2	1937·7	281·4	0·53	0·05	0·36
MAX d*P*/d*t* (mmHg/s)											
8·8 nM	2279·4	341·1	2613·7	203·9	2364·6	288·1	1521·2	153·4	0·07	0·35	0·04
70 nM	2624·4	563·3	2545·2	362·7	2538·8	175·1	2822·6	244·4	0·66	0·65	0·72
560 nM	2700·2	725·8	2618·4	350·0	2569·6	184·6	2983·0	510·8	0·81	0·73	0·61
AUC d*P*/d*t* (mmHg/s × s)											
8·8 nM	226 290·2	26 814·1	264 895·7	14 793·1	241 657·4	15 415·0	291 122·3	30 246·0	0·38	0·07	0·82
70 nM	245 046·0	26 004·0	262 897·7	22 634·4	258 241·7	13 357·0	337 422·1	25 346·0	0·06	0·04	0·17
560 nM	245 173·2	31 883·2	283 704·8	23 649·6	277 411·7	15 512·1	347 198·0	35 566·7	0·08	0·05	0·56

MLP, maternal low protein; MAX, maximum response; HR, heart rate; AUC, area under the curve; LVDP, left ventricular developed pressure; % LVDP, LVDP as a percentage increase from baseline with baseline set at 100 %; d*P*/d*t*, rate of pressure development.
* Doses of isoproterenol shown are selected from a dose range of 1·1–560 nM that included fourteen separate doses.

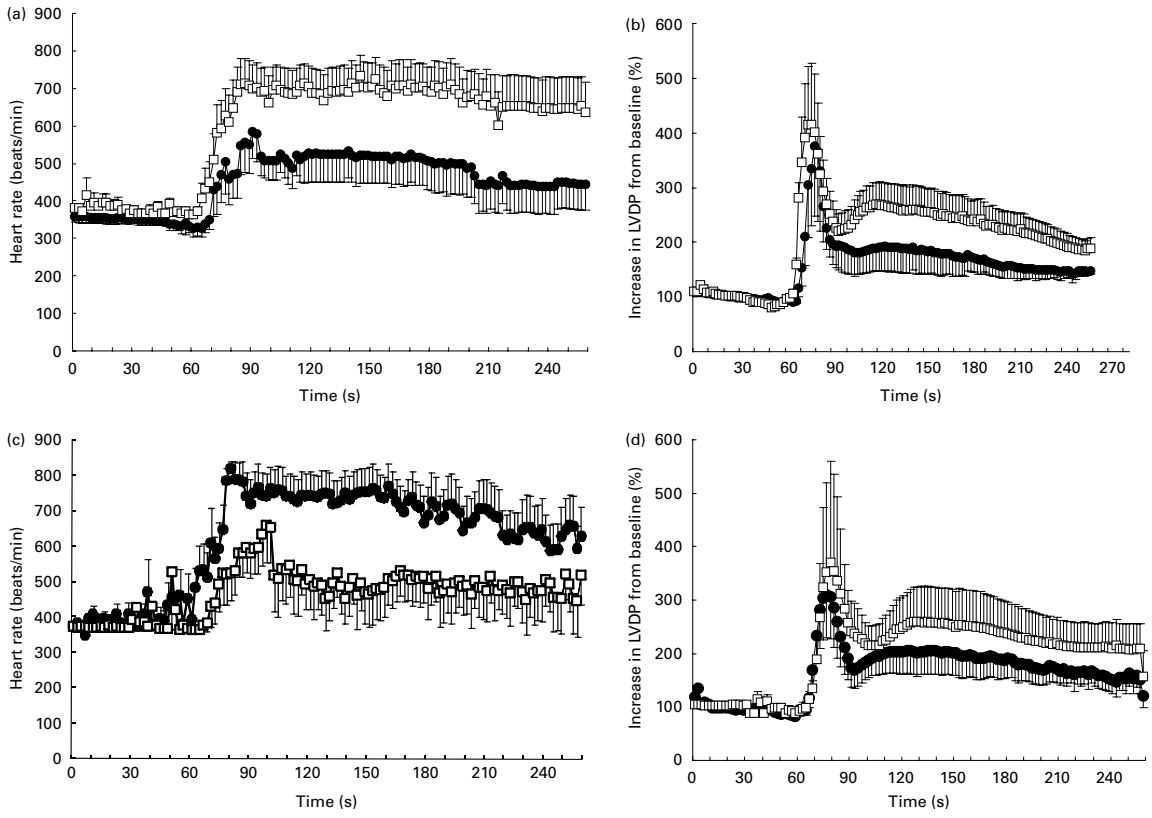

Fig. 1. The effects of a prenatal control (n 6; ●) or low-protein (n 6; □) diet on the heart rate (a, c) and left ventricular developed pressure (LVDP) response (b, d) to 560 nM-isoproterenol in male (a, b) and female (c, d) offspring. Values are means, with standard errors represented by vertical bars. A significant interaction between sex and diet was observed in maximum heart rate reached (P=0·02) and return to baseline (P<0·01). There was no significant effect of sex or diet on maximal LVDP response or return to baseline.

AUC for the male hearts (Fig. 2 (c) and Table 3), we noted differences in EC_{50} and Hill slope that approached statistical significance (P=0·058).

The effects of different concentrations of isoproterenol on inotropic (contractile) responses of the Langendorff perfused heart can be seen in the LVDP dose–response curves (Fig. 3 and Table 4). Control males were shown to be less sensitive to isoproterenol stimulation than male offspring of protein-restricted rats. Control rats had a larger EC_{50} and lower Hill slope (P=0·015) (Fig. 3 (a) and Table 4). In hearts from female rats both log EC_{50} and Hill slope values were similar in the two maternal dietary groups (Fig. 3 (b) and Table 4). As shown in Fig. 3 (c) and (d) and Table 4, the observed differences in return to baseline, noted above, did not significantly influence the EC_{50} and Hill slope values calculated from the AUC data.

Myocardial β_1- and β_2-adrenergic receptor mRNA expression

In hearts collected from rats at age 2 weeks, the expression of mRNA for β-actin and β_1-adrenergic receptors was not influenced by diet or sex. In contrast, myocardial expression of β_2-adrenergic receptors (Fig. 4) was influenced by diet (P=0·049), where gene expression was significantly increased in female animals following prenatal protein restriction.

Discussion

The findings of the present study clearly demonstrate that prenatal protein restriction alters the response of the isolated adult rat heart to β-agonist stimulation. The inotropic and chronotropic responses to isoproterenol following protein restriction *in utero* were generally unchanged in females, but significantly higher in males. This suggests that a MLP diet throughout gestation programmes the sensitivity of the adult rat heart's β-adrenergic receptor stimulation in a sex-specific manner. This is entirely consistent with our earlier finding that only male offspring of protein-restricted mothers have programmed vulnerability to ischaemia–reperfusion injury[7]. This is not the only investigation to document sex differences in cardiac function in response to β-adrenergic stimulation. Schwertz *et al.*[17] provided evidence that left atrial preparations from male rats had a greater response to adrenergic stimulation than those from females. These findings could be explained by sex differences in the cardiac tissue expression of β-adrenergic receptors. Male rat myocytes have been reported to have a 2-fold greater density of β-receptors and enhanced response to stimulation in comparison with myocytes isolated from female hearts[18].

Oestrogen plays a key role in the expression of β-adrenergic receptors of the heart, which subsequently control the cardiac response to sympathetic stimulation. Expression of β-receptors is up-regulated in the ovariectomised rat heart but quickly reversed following oestrogen replacement[19]. This cardioprotective effect of oestrogen may explain why the response to a sympathetic agonist in the female rat heart was not altered by prenatal protein restriction, whereas the cardiac response in their male littermates was.

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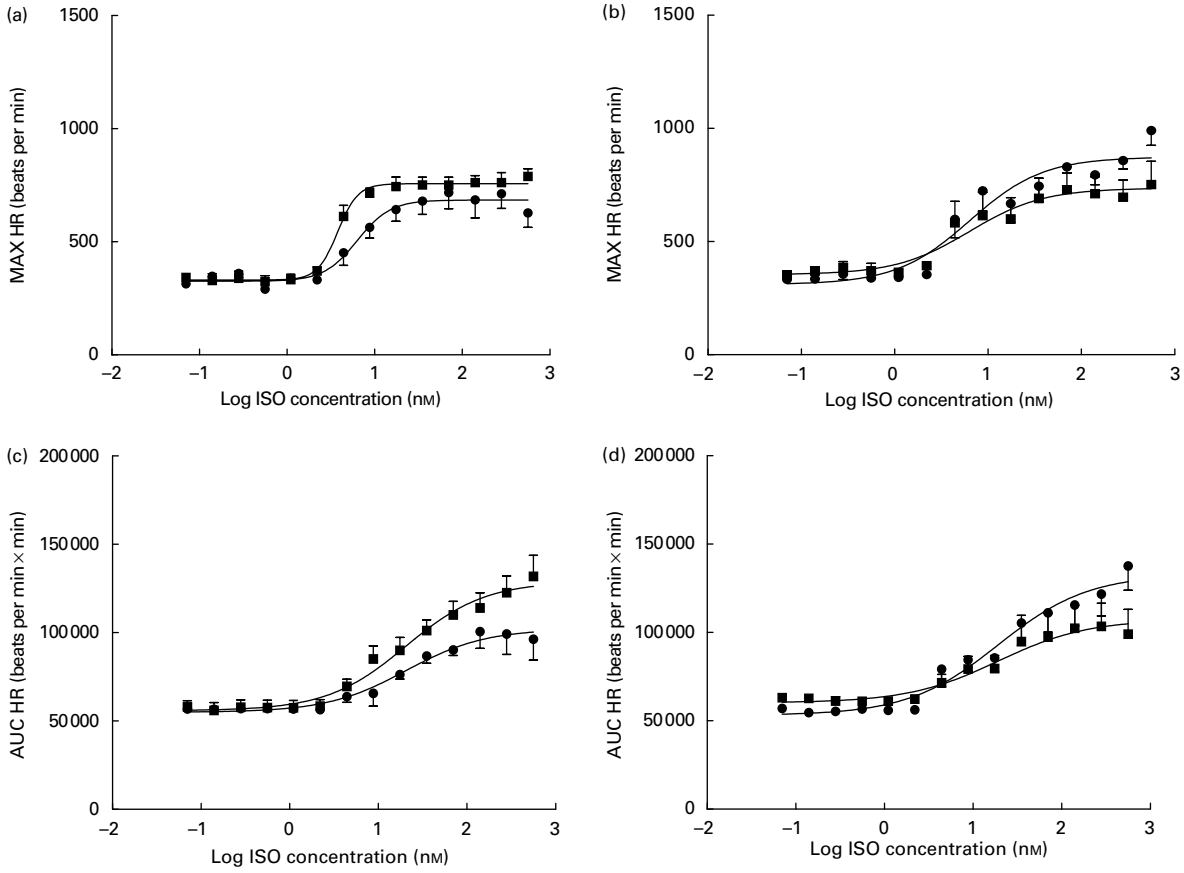

Fig. 2. Effect of different concentrations of isoproterenol (ISO) on contraction rate of the Langendorff perfused heart. Concentration dose–response curves represent the chronotropic response (a and b) normalised between dietary groups to compare the top and bottom of the curve, log EC_{50} and Hill slope; the lowest and highest values were taken as the bottom and top of the curve respectively (see Table 3). (a) Maximum heart rate (MAX HR) of male rats exposed prenatally to a control (●) or protein-restricted (■) diet. (b) MAX HR of female rats exposed prenatally to a control or protein-restricted diet. (c) Area under the curve (AUC) for control and protein-restricted male rats to compare the top and bottom of the curve, log EC_{50} and the curve gradient (see Table 3). (d) AUC for control and protein-restricted female rats. Curves are plotted as the mean and standard error for six individual curves per group.

Table 3. Contraction rate of the Langendorff perfused heart: slope parameters†
(Mean values and standard errors)

	Males				Females			
	Control (*n* 6)		MLP (*n* 6)		Control (*n* 6)		MLP (*n* 6)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
MAX HR (beats per min)								
Top	684·6	0·05	756·8	6·1	904·2	60·6	717·7	20·5
Bottom	325·6	14·8	331·1	7·1	302·9	53·7	355·8	22·3
Log EC_{50}	0·8	0·06	0·58	0·02	0·87	0·17	0·71	0·10
Hill slope	2·28	0·61	3·77	0·54	0·93	0·35	1·40	0·43
*P**	0·002				0·722			
AUC HR (beats per min × min)								
Top	99 212	1610	132 870	5722	141 672	12 832	102 546	2154
Bottom	56 575	967	54 321	2505	51 450	4782	61 336	1471
Log EC_{50}	1·30	0·05	1·36	0·10	1·50	0·26	1·15	0·08
Hill slope	1·42	0·21	0·82	0·15	0·64	0·20	1·27	0·27
*P**	0·0581				0·101			

MLP, maternal low protein; MAX, maximum response; HR, heart rate; AUC, area under the curve.
* *P* values refer to log EC_{50} and Hill slope.
† Slope parameters of dose–response curves shown in Fig. 2 (a) and (c) for hearts from male rats and Fig. 2 (b) and (d) for hearts from female rats.

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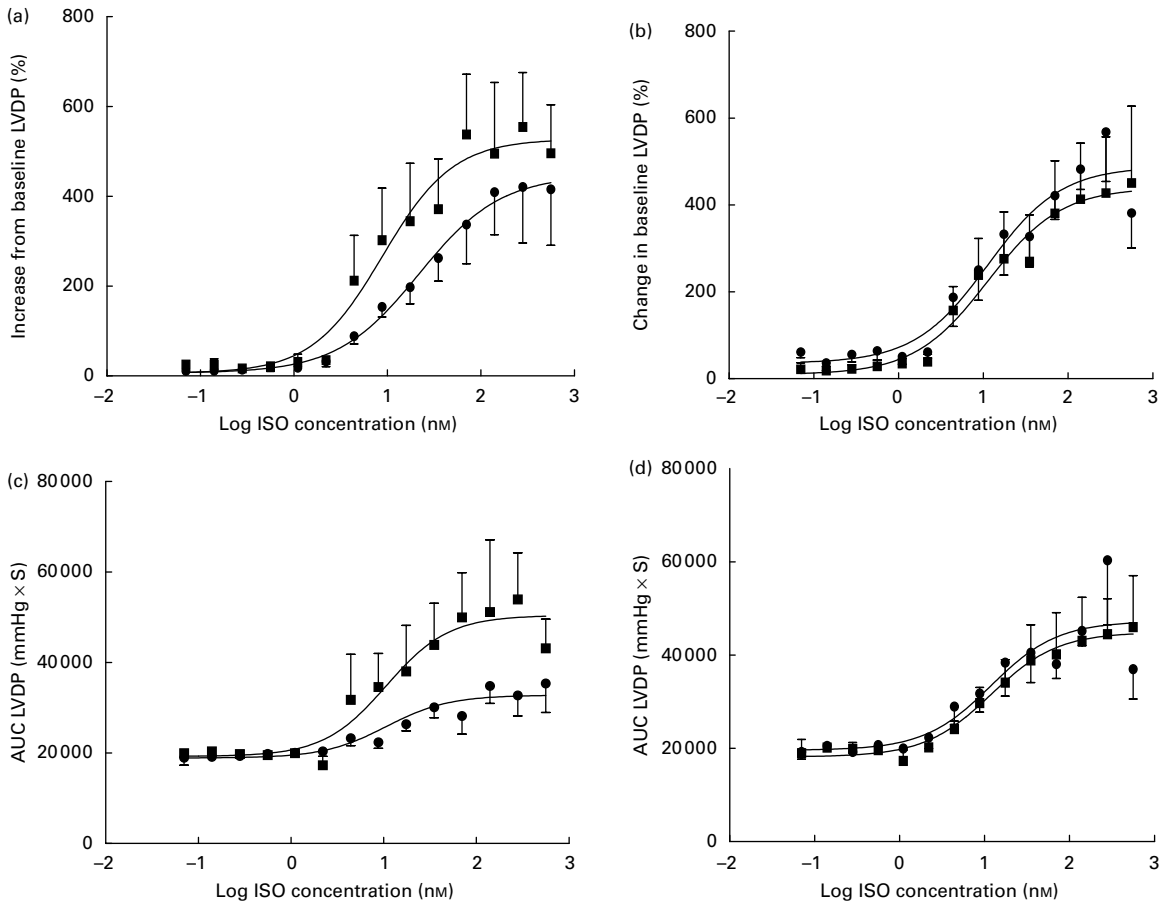

Fig. 3. Effect of different concentrations of isoproterenol (ISO) on left ventricular developed pressure (LVDP) on the Langendorff perfused heart. Concentration dose–response curves represent the inotropic responses as a percentage of baseline values (a and b) normalised between dietary groups to compare the top and bottom of the curve, log EC_{50} and Hill slope; the lowest and highest values were taken as the bottom and top of the curve respectively (see Table 4). (a) Increase in LVDP of male rats exposed prenatally to a control (●) or protein-restricted (■) diet. (b) Increase in LVDP of female rats exposed prenatally to a control or protein-restricted diet. (c) Area under the curve (AUC) for control and protein-restricted male rats to compare the top and bottom of the curve, log EC_{50} and Hill slope (see Table 4). (d) AUC for control and protein-restricted female rats. Curves are plotted as the mean and standard error for six individual curves per group.

Table 4. Inotropic responses of the Langendorff perfused heart: slope parameters†
(Mean values and standard errors)

	Males				Females			
	Control (n 6)		MLP (n 6)		Control (n 6)		MLP (n 6)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Increase from baseline LVDP (%)								
Top	447·1	15·8	527·6	29·9	477·8	40·2	459·1	33·2
Bottom	6·35	7	5·95	5·2	40·7	30·4	1·7	2·73
Log EC_{50}	1·34	0·05	0·95	0·1	1·03	0·15	1·1	0·12
Hill slope	0·99	0·01	1·15	0·3	1·14	0·41	0·85	0·19
P*	0·0145				0·745			
AUC LVDP (mmHg × s)								
Top	36 304	2459	49 699	2300	47 044	4811	44 976	1015
Bottom	18 904	942	19 110	1929	18 953	3697	18 724	698
Log EC_{50}	1·44	0·2	0·97	0·12	1·0	0·29	1·13	0·06
Hill slope	0·81	0·26	1·38	0·49	1·03	0·67	1·22	0·18
P*	0·0994				0·799			

MLP, maternal low protein; LVDP, left ventricular developed pressure; AUC, area under the curve.
* P values refer to log EC_{50} and Hill slope.
† Slope parameters of dose–response curves shown in Fig. 3 (a) and (c) for hearts from male rats and Fig. 3 (b) and (d) for hearts from female rats.

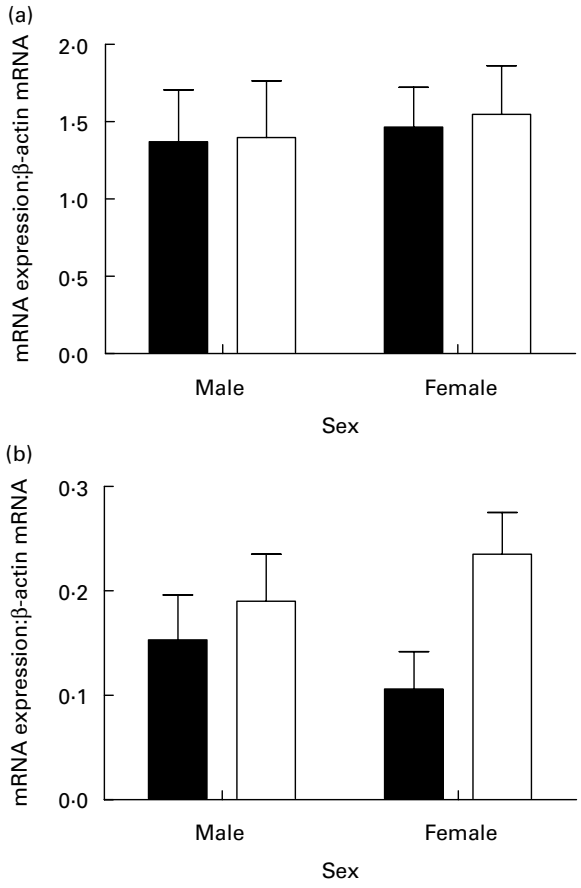

Fig. 4. The effect of a prenatal control (■) or low-protein (□) diet on the myocardial expression of β_1- (a) and β_2- (b) adrenergic receptors in offspring at age 2 weeks. Data are means for seven to eleven observations, with standard errors represented by vertical bars. Expression of mRNA for β-actin and β_1-receptors was not influenced by diet or sex. Expression of mRNA for β_2 was influenced by diet ($P=0{\cdot}049$) only.

Cardiac muscle reactivity towards β-adrenergic stimulation has been documented to be increased following food restriction. Klebanov *et al.* [15] determined an increased sensitivity to low-perfusate Ca and isoproterenol in isolated rat hearts that had been submitted to long-term food restriction. A similar study showed that Langendorff-perfused rat hearts have an enhanced response to isoproterenol following long-term food restriction[20]. Although these effects of postnatal undernutrition are well established, to date, only one other study has looked at the cardiac β-adrenergic response in rats subjected to prenatal protein restriction. Fernandez-Twinn *et al.* [21] showed *in vivo* that in hearts of males exposed to low-protein diets throughout fetal life and suckling, chronotropic responses to a low dose of isoproterenol were blunted. In addition there was a delay in hearts reaching their maximal response. Following protein restriction in early life, the observed response was shorter in duration but exhibited a delay in the complete return to baseline. The maximal inotropic response to a high dose, as measured by changes in mean arterial pressure, was similar between the two dietary groups. However, in offspring of protein-restricted rats, the maximal cardiac response was achieved in significantly less time than controls, although the duration of the maximal response was reduced. It was concluded from these results that hearts from low-protein-exposed offspring have decreased adrenergic signalling due to a reduced β-adrenergic response to isoproterenol. This conclusion is in complete contrast to the present study, where we provide evidence that both chronotropic and inotropic responses of the male rat heart to isoproterenol treatment were enhanced following prenatal protein restriction.

A delay in recovery to basal cardiac function following isoproterenol treatment was an interesting observation common to the present study and the work of Fernandez-Twinn *et al.* [21]. However, direct comparisons cannot be made between these two studies, which differ in many aspects. Although both experiments utilised low-protein diets, the full composition of the diets differed. Whilst our diet provided carbohydrate as a mixture of starch and sucrose, that used by Fernandez-Twinn *et al.* provided mostly glucose. Moreover, the present study provided 10 % fat by weight, with all fat in the form of maize oil. In contrast the Fernandez-Twinn diet delivered 3·9 % fat as soya oil. The present study focused solely on protein restriction in pregnancy, whilst the previous work[21] examined the impact of undernutrition in pregnancy and lactation. Nutritional insults may exert contrasting effects when applied during different critical periods of development. Other key differences between the two studies are the variation in doses of isoproterenol and the age of the animals used. Most importantly, whilst the present study utilised an *ex vivo* model of cardiac function, Fernandez-Twinn *et al.* examined function *in vivo*, using chronically cannulated animals.

One technical advantage that the *ex vivo* isolated heart model used in the present study has over *in vivo* heart models[21] is that data measurements can made without the influence of other organs and the systemic circulation, and in particular the release of circulatory hormones. It could be argued that a cardiomyocyte cell-culture model would be better to characterise the β-adrenergic receptor's responsiveness to isoproterenol in MLP-fed offspring. However, at the present time there is insufficient evidence to guarantee that the cells in culture retain a cellular memory of the programming events in fetal life.

The myocardial β-adrenergic receptor signalling pathway plays a crucial role in the development of IHD. Sympathetic hyperactivity has been shown to be a causal factor in the development of adult hypertension and the common strategy to decrease high blood pressure includes the therapeutic use of β-adrenergic antagonists. Increased heart rate[21], adult hypertension[5,22] and greater cardiac dysfunction following ischaemia–reperfusion injury[7] have all been found in animal studies where fetal development has been challenged nutritionally. These findings suggest programmed changes in the cardiac β-adrenergic signalling pathway in offspring of nutrient-restricted animals. The present study strongly supports this view, as isolated hearts from prenatal protein-restricted male rats showed a greater response to the non-specific β-agonist isoproterenol. This is of interest as greater stimulation of the cardiac β-adrenergic system has been shown to contribute to the pathology of congestive heart failure[23].

An important finding of the present study is that the myocardial expression of mRNA for β-adrenergic receptors was significantly different in hearts from 2-week-old offspring subjected to control or MLP diets *in utero*. The β_1-adrenergic

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receptors were not influenced by sex or maternal diet. In contrast, the β_2-adrenergic receptors were expressed at a higher level following exposure to a MLP diet *in utero* in females only. These changes were observed at a developmental stage that precedes previously reported hypertension and may therefore be suggested to have some causal role.

Over-expression of cardiac β_2-adrenergic receptors have been shown to enhance myocardial contractility and relaxation, which becomes unresponsive to further isoproterenol stimulation[24]. Administration of β_2-adrenergic receptor antagonists in the same model results in a dramatic fall in contractile function[25], thereby establishing that β_2-adrenergic receptor stimulation is a necessity for contractile function. Further work by Dorn *et al.*[26] recognised that low-level over-expression of cardiac β_2-adrenergic receptors generates beneficial effects, suggesting that an optimum expression of β_2-receptors exists to maintain cardiac function. This could be a key mechanism whereby female hearts from low-protein-exposed offspring, which exhibit greater expression of β_2-adrenergic receptors, are protected from the greater sensitivity to stimulation by isoproterenol that we observed in the male offspring.

Interestingly the mRNA expression of cardiac β-adrenergic receptors did not match changes seen at the protein level in the study by Fernandez-Twinn *et al.*[21]. This earlier study established that the expression of β_1-adrenergic receptors was reduced in offspring exposed to a MLP diet when compared with controls, and that it was the expression of β_2-adrenergic receptors that was unaffected. Although these findings are in complete contrast to each other it is entirely possible that expression of protein does not correspond to expression of mRNA. Further studies are required to investigate this discrepancy, and should also consider programming effects upon receptor density, affinity for agonists and activity of second messenger systems. Changes in any one, or all of these components of receptor function could be responsible for changes in β-adrenergic responsiveness. In line with our data, cardiac over-expression of β_2-adrenergic receptors in male mice results in increased contractility and increased ischaemic–reperfusion injury[27]. In addition, recent work in GM mice has shown that over-expression of cardiac β_2-adrenergic receptors enhances contractility without any pathological consequences[28], but by comparison reducing expression of the β_1-adrenergic receptor produces significant ventricular dysfunction[29]. It was concluded from these findings that functional differences between β_1- and β_2-adrenergic receptors exist and that a mild or defined stimulation in cardiac contractility is potentially beneficial to the diseased myocardium. These discoveries are consistent with our finding that MLP-exposed male rats are more susceptible to cardiac injury[7], and adds weight to our argument that risk of cardiac dysfunction may be explained by altered β-receptor expression. Female mice are relatively protected from the detrimental effects of β_2-adrenergic receptor over-expression[27]. This could explain why, in the present study, although programmed over-expression of β_2-receptor expression appeared greater in females than in males, functional markers of cardiac responsiveness to the agonist were only programmed in males.

Catecholamines are essential for myocardial function and emphasis is placed on the role they play in adrenergic receptor signal transduction. Increased β-adrenergic receptor stimulation has been shown to have detrimental effects on the heart and is a major cause of heart failure[30–32]. An increase in circulating catecholamine levels during heart failure decreases cell-surface β-adrenergic receptors as a result of down-regulation[33]. A decline in the number of myocardial β_1-adrenergic receptors is associated with increased responsiveness to β_2-receptor stimulation[34]. It has been established that circulating concentrations of the catecholamines adrenaline and noradrenaline are higher in adult offspring fed a reduced-protein diet throughout pregnancy and lactation[35], or those that underwent placental restriction during fetal development[36]. Increased circulating levels of catecholamines could be considered a possible factor in the development of adult hypertension in these models, and could explain the findings of the present study where protein-restricted male offspring exhibited a greater response to the β-agonist isoproterenol. Although accurate determination of catecholamine concentrations *in vivo* is problematic, such measurements may be a useful element of future work in this model.

Our recent study has shown that prenatal protein restriction impairs the recovery of the adult rat heart to ischaemia–reperfusion injury in a sex-specific manner[7]. Myocardial ischaemia activates the efferent sympathetic nerve activity to the heart and accelerates glycogenolysis to increase energy production. Increasing sympathetic nerve activity may be favourable for the production of energy during short periods of ischaemia, but may be harmful to the heart and produce irreversible myocardial damage during longer periods[37]. We argue that it is entirely plausible that the greater cardiac sensitivity to β-agonists in male offspring predisposes them to cardiac dysfunction, especially during an ischaemic event. In conclusion, the present study provides evidence that prenatal protein restriction programmes the cardiac responses to β-adrenergic agonists, in a sex-specific manner. Following protein restriction *in utero*, male hearts are more sensitive to β-adrenergic receptor stimulation. This greater sensitivity to β-adrenergic agonists in adult male hearts from protein-restricted offspring may play an important role in the programmed development of hypertension and myocardial dysfunction following ischaemia–reperfusion.

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