Maternal undernutrition programmes atherosclerosis in the ApoE*3-Leiden mouse

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Poor quality of nutrition during fetal development is associated with adverse health outcomes in adult life. Epidemiological studies suggest that markers of fetal undernutrition are predictive of risk of the metabolic syndrome and CHD. Here we show that feeding a low-protein diet during pregnancy programmed the development of atherosclerosis in ApoE*3-Leiden mice. ApoE*3-Leiden mice carry a mutation of human ApoE*3 rendering them prone to atherosclerosis when fed a diet rich in cholesterol. It was noted that fetal exposure to protein restriction led to a greater degree of dyslipidaemia in mice when fed an atherogenic diet, with low-protein-exposed ApoE*3 mice having elevated total plasma cholesterol (34 % higher; $P < 0.001$) and TAG (39 % higher; $P < 0.001$) relative to offspring exposed to a control diet *in utero*. The low-protein group developed more severe atherosclerotic lesions within the aortic arch (2·61-fold greater lesion area; $P < 0.001$). Analysis of a targeted gene array suggested a potential role for members of the LDL receptor superfamily, along with similar programmed suppression of the mRNA expression of hepatic sterol regulatory element-binding protein-1c. This indicates that disordered lipid metabolism may play a role in the fetal programming of atherosclerosis in this model. Whereas earlier studies have shown early programming of cardiovascular risk factors, these results demonstrate for the first time that the interaction of prenatal undernutrition with a postnatal atherogenic diet increases the extent of atherosclerotic disease.

Atherosclerosis: Lipid metabolism: Protein restriction: Programming

It is acknowledged that the onset and development of disease in adult life is associated with quantity and quality of nutrition during the fetal period[1]. Epidemiological studies in developed and developing countries have strongly suggested that the intra-uterine environment plays a role in determining risk of adult disease[2,3]. Many cohort studies indicate that lower weight at birth, followed by rapid catch-up growth in childhood, is associated with risk of the metabolic syndrome and CVD in later life. It has been proposed that maternal undernutrition may 'programme' long-term changes in gene expression and therefore metabolism in the fetus, resulting in cardiovascular abnormalities in later life[4]. While the origins of the metabolic syndrome are multifactorial, maternal nutrition and its impact during fetal development may be an important contributing factor. Work from Napoli and colleagues has, for example, demonstrated that atherosclerotic lesions begin to form during fetal life, in both humans and animals, and that this process is accelerated by maternal hypercholesterolaemia[5,6]. Moreover the expression of genes that predispose to, or protect against, these conditions will be further modified by interactions between the genotype, early life nutrition and the postnatal environment[7].

Transgenic mice with an altered lipoprotein metabolism, in particular transgenic and knockout mice based on the *ApoE* gene, have been important tools for the elucidation of the relationships between hyperlipidaemia and atherosclerosis.

The ApoE*3-Leiden mouse carries a naturally occurring tandem duplication mutation of codons 120–126 in the human *ApoE* gene, on a C57Bl/6J background[8]. This results in impaired clearance of lipoproteins from the plasma, raised plasma lipid levels and a greater susceptibility to developing atherosclerosis when the mice are fed diets rich in cholesterol[9]. Whilst other transgenic mouse strains, for example the LDL receptor (LDLr) knockout mouse, develop atherosclerosis even when fed a standard chow diet, the cholesterol-rich diet is an absolute requirement for the development of lesions in the ApoE*3-Leiden mouse. This makes this strain ideal for studies evaluating the influence of diet upon atherosclerosis and CHD.

Less than optimal intakes of protein remain commonplace throughout the world, impacting upon populations in developing countries and among the lower socio-economic groups in developed nations. In the rat, fetal exposure to a maternal low-protein diet has been consistently shown to programme high blood pressure, impairments of renal function, dyslipidaemia and glucose intolerance[7]. Although these phenotypes are commonly seen in the offspring of rodents and sheep subject to a variety of different manipulations of the maternal diet, they are all risk factors for disease rather than disease outcomes in their own right. In the present study, therefore, we aimed to assess the capacity of undernutrition to programme atherosclerosis using the well-established low-protein model.

Materials and methods

Animal protocols

All experiments involving mice were performed in accordance with the Animals (Scientific Procedures) Act 1986 and subject to UK Home Office regulations. Male and female mice (aged 10–12 weeks) were maintained in a controlled environment (21°C; 55 % humidity) with a 12 h light–dark cycle. Animals were maintained on a standard laboratory chow diet (Beekay Universal, Hull, UK) and had *ad libitum* access to food and water at all times. Male ApoE*3-Leiden transgenic mice, on a C57BL/6J background, were mated with wild-type C57BL/6J females. The ApoE*3-Leiden transgene is lethal to homozygotes, so this mating strategy was necessary to produce mice that were heterozygous for the transgene, and which would therefore be atherosclerosis-prone. All litters in the study therefore contained a mixed population of wild-type and transgenic offspring. The pregnant females were fed either a control (18 % casein; *n* 20) or a low-protein (9 % casein; *n* 22) diet, as described previously[10]. At birth all mothers were transferred to the same standard chow diet. The offspring therefore differed only in their prenatal dietary exposures. Mothers and offspring were otherwise left undisturbed until weaning, as preliminary work with these mice suggested that handled pups may be rejected by their mothers. Offspring were genotyped using PCR before weaning at 28 d postnatal age[11]. Based on their genotype, sex and prenatal experience, offspring were then allocated to be fed either a chow diet or an atherogenic diet comprising 15 % cocoa butter, 40·5 % sucrose and 0·25 % cholesterol. The latter was designed to induce the disease process, as in the ApoE*3-Leiden mice, cholesterol in the diet produces proportionate increases in circulating cholesterol[9]. There were eight treatment groups of male and female offspring from both control and low-protein-fed mothers. Offspring were of either wild-type or ApoE*3-Leiden genotype. As, in keeping with previous studies of ApoE*3-Leiden mice[12], we observed neither significant hypercholesterolaemia, nor atherosclerosis in male offspring fed the atherogenic diet, we report here only the data from the female offspring in the trial.

After 3 months of postnatal feeding, animals were killed using a rising concentration of carbon dioxide and were not fasted before cull. Whole blood was collected into vacutainers by heart puncture and plasma prepared by centrifugation at 13 000 rpm at 4°C for 10 min. The liver, adipose (perirenal and gonadal depots), kidneys and abdominal aorta were dissected from each animal, weighed to the nearest 0·1 mg and snap-frozen in liquid N_2. Hearts and the aortic root were dissected from each animal and infused with OCT fixing compound (Miles Inc., Elkhart, IN, USA) and snap-frozen in OCT until sectioning.

Genotyping of transgenic mice

Genomic DNA was extracted from 0·3 cm of mouse tail by standard procedures[10]. PCR was performed on genomic tail DNA using primers spanning the ApoE*3-Leiden mutation (forward primer 5′ GCCCCGGCCTGGTACACTGC 3′; reverse primer 5′ GGCACGGCTGTCCAAGGAGC 3′).

Measurement of plasma metabolites

Total circulating plasma cholesterol and TAG were assayed using commercially available kits (ThermoTrace, Noble Park, Vic, Australia), according to the manufacturer's instructions. Assay linearity was 20 mmol/l for cholesterol and 10 mmol/l for TAG; assay sensitivity was 62 ΔmA per mmol/l for cholesterol and 0·158 ΔA per mmol/l for TAG.

Histological analysis of the heart and aortic root

Frozen heart and aortic root samples were sectioned using a cryostat (Bright Instruments, Huntingdon, Cambs, UK). Alternate sections of 10 μm thickness were collected of the aortic root, stained with Oil Red O and imaged using a Nikon phase contrast 2 microscope and a MicroPublisher 3·3 RTV camera (Q Imaging, St Helens, Lancs, UK). Atherosclerotic lesions were analysed and quantified following the method of Paigen *et al.*[13] using Image Pro-Plus software (Media Cybernetics, Inc., Bethesda, MD, USA) to determine the percentage of the total area of the aortic intima exhibiting atherosclerotic lesions. The average lesion area for each animal was calculated using data from fifteen sections per animal.

Oligo GEArray® analysis of gene expression

In order to assess some of the mechanisms that might lead offspring of low-protein-fed mice to be more prone to atherosclerosis in postnatal life, we used a targeted DNA microarray to analyse transcripts in the liver. Liver was selected as the main tissue of interest, as earlier work with rats[7] suggested programming of disturbed lipid metabolism could be of particular significance in the ApoE*3-Leiden mouse. RNA was extracted from the livers of ApoE*3-Leiden female mice using the TRIzol® method (Invitrogen Corp., Carlsbad, CA, USA). RNA was quantified on a NanoDrop® spectrophotometer (ND-1000; NanoDrop®, Wilmington, DE, USA) and ribosomal band integrity was assessed on an agarose gel and an Agilent Bioanalyzer® (Agilent Technologies, Inc., Santa Clara, CA, USA). cDNA and cRNA were synthesised and the latter labelled using the SuperArray TrueLabelling-AMP™ 2·0 kit (according to the manufacturer's instructions). Target cRNA was hybridised to each microarray using the Oligo GEArray® System (Super-Array; SABiosciences, Frederick, MD, USA), according to the manufacturer's guidelines. Four to six microarrays were used for each group, with RNA from one randomly selected mouse per group used per array. Microarrays were exposed to X-ray film for 30 s, 1 min, 2 min and 5 min to identify the exposure time which produced the largest possible dynamic range in the individual signals. Images were captured using a Fluor-S multi-imager and saved as 16 bit TIFF images. Image analysis and data acquisition were performed using the GEArray Expression Analysis Suite (www.sabiosciences.com; SABiosciences, Inc.). The full list of genes included in the array is shown in Table 1. Data were normalised to the arithmetic mean of the housekeeping genes, Rps27a (ribosomal protein S27a), B2m (β-2 microglobulin), Hspcb (heat-shock protein 1 β) and Ppia (peptidylprolyl isomerase A).

Determination of mRNA expression

Quantitative PCR was performed as a follow up to the microarray studies. Hepatic RNA was extracted using the TRIzol®

Table 1. Full list of genes that were included in the microarray analysis

	Genes
Mediators of the response to stress	
Inflammatory response	Apoa2, Ccl11, Ccl2, Ccl20, Ccl5, Ccr1, Ccr2, Cxcl1, Ifng, Il1a, Il1b, Il2, Itgb2, Pparg, Selp, Spp1, Tgfb1, Tnf
Response to pest, pathogen or parasite	Fn1, Il10, Il2, Il4, Il6, Spp1
Other genes related to the stress response	Apoe, Bax, Bcl2l1, Sod1, Sod2
Apoptosis	
Anti-apoptosis	Bcl2, Bcl2l1, Birc3, Il10, Spp1, Vegfa
Induction of apoptosis	Apoe, Bax, Tnfrsf6
Other genes related to apoptosis	Bcl2a1a, Bid, Cflar, Ifng, Il6, Nfkb1, Sod1, Tnfaip3
Blood coagulation and circulation	Apoe, F7, Npy, Ptgs1, Ptgs2, Vwf
Adhesion molecules	
Cell−cell adhesion	Cdh5, Icam1, Icam2, Vcam1
Cell−matrix adhesion	Ctgf, Itga2, Itga5, Itgax, Itgb2, Itgb3, Itgb5, Itgb7, Spp1
Other genes involved in adhesion	Cd36, Cd44, Eng, Fn1, Lama1, Scarb1, Sele, Sell, Selp, Selpl, Snn, Thbs4
ECM molecules	
ECM protease inhibitors	F7, Serpinb2, Serpine1
ECM proteases	Ace, F7, Mmp13, Mmp1a, Mmp3, Mmp9, Serpinb2, Serpine1
ECM structural constituents	Col3a1, Eln, Lama1
Other extracellular molecules	Apoa1, Apoa2, Apoa4, Apoe, Ccl11, Ccl2, Ccl20, Ccl5, Cdh5, Csf1, Csf2, Csf3, Ctgf, Cxcl1, Dtr, Eng, Fga, Fgb, Fgf2, Fn1, Icam2, Ifnar2, fng, Il10, Il13, Il1a, Il1b, Il1r1, Il1r2, Il1rl1, Il2, Il3, Il4, Il5, Il6, Il7, Itga2, Itga5, Itgb2, Itgb5, Itgb7, Kdr, Lcat, Ldlr, Lif, Lpl, Npy, Pdgfa, Pdgfb, Pdgfrb, Ptgs1, Ptgs2, Sele, Selp, Selpl, Spp1, Tgfb1, Tgfb2,Tgfb3, Thbs4, Tnc, Vcam1, Vegfa, Vwf
Lipid transport and metabolism	
Cholesterol metabolism	Abca1, Apoa1, Apoa2, Apoa4, Apoe, Il4, Lcat, Ldlr, Soat2
Fatty acid metabolism	Apoa2, Apob, Lypla1, Ppara, Ptgs1, Ptgs2
Lipid transport	Abca1, Adfp, Apoa1, Apoa2, Apoa4, Apob, Apoe, Fabp3, Ldlr, Lpl, Msr1
Lipoprotein metabolism	Abca1, Apoa1, Apoa2, Apoa4, Apoe, Ldlr, Lpl, Msr1, Olr1
Steroid metabolism	Nr1h3, Ppara, Ppard, Pparg, Rxra, Soat2
Cell growth and proliferation	
Growth factors and receptors	Csf1, Csf2, Csf3, Ctgf, Cxcl1, Dtr, Fgf2, Il10, Il1a, Il1b, Il2, Il3, Il4, Il5, Il6, Il7, Kdr, Lif, Pdgfa, Pdgfb, Pdgfrb, Spp1, Tgfb1, Tgfb2, Tgfb3, Vegfa
Regulation of the cell cycle	Fgf2, Il1a, Il1b, Pdgfa, Pdgfb, Tgfb1, Tgfb2, Tgfb3, Vegfa
Other genes involved in cell growth and proliferation	Eln, Eng, Fn1, Ifnar2, Ifng, Itga5, Itgb3, Ppard
Regulators of transcription	
Nuclear receptors	Nr1h3, Ppara, Ppard, Pparg, Rxra
Other regulators of transcription	Ccl5, Egr1, Ifnar2, Ifng, Klf2, Nfkb1, Sod2

ECM, extracellular matrix.

method (Invitrogen Corp.) according to the manufacturer's guidelines. cDNA was synthesised using MML-V RT (Promega Corp., Madison, WI, USA) and quantitative RT-PCR was performed using a Roche Light Cycler 480® (Roche Diagnostics, Basel, Switzerland). Flurogenic probes were labelled with 6-carboxy-fluorescin (FAM) at the 5′ end and with 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′ end. A negative template control and a relative standard curve were included on every PCR run. The standard curve was prepared from a pool of sample cDNA over a range of dilutions. Relative target quantity was calculated from the standard curve and all samples were normalised against the geometric mean of four house-keeping genes, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 36B4 and hypoxanthine phosphoribosyl transferase (HPRT)[14]. Sequences of primers and probes used for RT-PCR are shown in Table 2.

Statistical analysis

All data are presented as mean values with their standard errors. Unless stated otherwise in the text, data were analysed using a mixed-model analysis using SPSS (version 14.0; SPSS, Inc., Chicago, IL, USA). In the case of plasma TAG,

cholesterol and mean atherosclerotic lesion area maternal diet, postnatal diet and genotype were the fixed factors and the results adjusted for within-litter effects[15]. This adjustment removed the influence of having littermates within some of the groups and is an analytical approach we have used in our previous studies of programming[16,17]. For microarray gene expression data and quantitative RT-PCR expression data, where only ApoE*3-Leiden offspring were studied, maternal diet and postnatal diet were the fixed factors and the results adjusted for within-litter effects. It was not possible to identify differences between specific groups where the difference may have arisen through an interaction of two or more factors. *Post hoc* tests were not performed where ANOVA indicated interactive effects.

Results

Pregnant C67Bl/6J mice fed control or low-protein diets gave birth to litters of similar size (control, 5·4 (SEM 0·4) pups per litter; low protein, 5·1 (SEM 0·4) pups per litter). The males:females ratio was similar in both prenatal dietary groups (control, 0·92; low protein, 0·90). The proportion of ApoE*3-Leiden mice produced by the pregnant mice was not

Table 2. Probe and primer sequences for real-time polymerase chain reaction studies

| Gene	Sequences 5′ to 3′	Temperature (°C)	Accession number
36B4	Forward: GCTTCATTGTGGGAGCAGACA	59·8	NM_007475
	Reverse: CATGGTGTTCTTGCCCATCAG	59·8	
	Probe: TGGGAGGCCATCACAATTGTGGC	66·1	
B-Actin			NM_007393
GAPDH	Forward: GAACATCATCCCTGCATCCA	65·9	DQ403055
	Reverse: CCAGTGAGCTTCCCGTTCA	66·5	
	Probe: CTTGCCCACAGCCTTGGCAGC	75·1	
HPRT	Forward: TTGCTCGAGATGTCATGAAGGA	58·4	NM_013556
	Reverse: TGAGAGATCATCTCCACCAATAACTT	60·1	
	Probe: TGGGAGGCCATCACAATTGTGGC	64·2	
Human apoE	Forward: CGTTGCTGGTCACATTCCTG	59·4	NM_000041
	Reverse: GCTGTCTCTCCACCGCTTG	61·0	
	Probe: CAGGATGCCAGGCCAAGGTGGA	65·8	
Mouse apoE	Forward: GCCCTGCTGTTGGTCACA	58·2	NM_009696
	Reverse: TGATCTGTCACCTCCGGCTC	61·4	
	Probe: TGCTGACAGGATGCCTAGCCGAGG	67·8	
LDLr	Forward: GCATCAGCTTGGACAAGGTGT	59·8	NM_010700
	Reverse: GGGAACAGCCACCATTGTTG	59·4	
	Probe: CACTCCTTGATGGGCTCATCCGACC	67·9	
LRP-1	Forward: TGGTCTGATGTGCGGACTCA	59·4	NM_008512
	Reverse: AACAGATTTCGGGAGACCCAG	59·8	
	Probe: TCTGCAGACTTGCCCAACGCCC	65·8	
SREBP-1a	Forward: AGGCGGCTCTGGAAACAGA	67·3	NM_011480
	Reverse: ATGTCGTTCAAACCGCTGTGT	66·6	
	Probe: TGGCCGAGATGTGCGAACTGGA	76·1	
SREBP-1c	Forward: ATCGGCGCGGAAGCTGTCGGGGTAGCGTC	85·5	NM_011480
	Reverse: ACTGTCTTGGTTGATGAGCTGGAGCAT	74·9	
	Probe: CGGAGCCATGATTGCACATTTGA	75·2	
SREBP-2	Forward: CAAGTCTGGCGTTCTGAGGAA	66·6	NM_033218
	Reverse: ATGTTCTCCTGGCGCAGCT	67·3	
	Probe: CCATTGATTACATCAATATCTGCAGCAGGTCAA	74·3	
VLDLr	Forward: GCGAGAGCCTGCCTCCA	60·0	NM_013703
	Reverse: CGCCCCAGTCTGACCAGT	60·5	
	Probe: CTGTGGATCCGTTGTCGGGCTTGT	66·3	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyl transferase; LDLr, LDL receptor; LRP-1, LDL receptor-related protein 1; SREBP, sterol regulatory element-binding protein; VLDLr, VLDL receptor.
* Supplied by Applied Biosystems (Foster City, CA, USA).

significantly different ($P > 0·05$; χ^2 test) in the two maternal dietary groups (control, 37·3 % transgenic; low protein, 33·8 % transgenic). Maternal food intake was similar in control and maternal low-protein pregnancies (data not shown). Offspring were not weighed at birth in order to avoid maternal stress and rejection of pups, but as shown in Fig. 1 (a), there were influences of maternal diet by the time the animals were weaned at 28 d. Offspring exposed to low-protein diets *in utero* were heavier ($P = 0·002$) than those from control diet-fed dams. There were no differences in weight between animals of different genotypes, and weights of animals allocated to postnatal chow or atherogenic diets were similar at the start of the feeding trial (Fig. 1 (a)). At the end of the 3-month feeding period the low-protein-exposed offspring remained heavier than the prenatal controls ($P = 0·002$), although this effect appeared to be confined to the C57Bl/6J strain (Fig. 1 (b)). At cull perirenal and gonadal fat pads were collected and carefully weighed. The sum of these fat pads, corrected for body weight, was used as a measure of abdominal fat deposition. It was noted that mice exposed to low-protein diet *in utero* had more fat at these sites than controls (effect of maternal diet; $P = 0·023$). Feeding of the postnatal atherogenic diet increased fat depot size relative to body weight in C57Bl/6J mice (Fig. 2), but this effect was not observed in the ApoE*3-Leiden mice (interaction of pre- and postnatal diets; $P = 0·002$).

The mice were culled after 3 months of postnatal feeding, for collection of blood and tissues. As shown in Fig. 3, the plasma cholesterol and TAG concentrations in the ApoE*3-Leiden mice were generally similar to wild-type C57Bl/6J offspring, when fed the chow diet. Females of the ApoE*3-Leiden strain developed a massive hypercholesterolaemic response to the atherogenic diet ($P < 0·001$). We observed an interactive effect of the maternal diet and postnatal diet ($P = 0·029$), indicating that ApoE*3-Leiden females exposed to the low-protein diet had higher total cholesterol concentrations following the atherogenic diet than those exposed to the control diet *in utero*. Plasma TAG concentrations were higher in female ApoE*3-Leiden mice than in wild-type animals. As with cholesterol, the response to the atherogenic diet was greater in the low-protein-exposed group than in the controls (genotype × maternal × postnatal diet interaction; $P = 0·047$).

The degree of atherosclerosis observed in female ApoE*3-Leiden offspring is shown in Fig. 4. Wild-type animals showed no effects of either maternal diet or atherogenic diet (data not shown). However, when we considered lesion area in the ApoE*3-Leiden females it was clear that the atherogenic diet induced lesions to a significantly greater extent (2·61-fold) in the animals exposed to a low-protein diet *in utero* than in those exposed to the control diet ($P = 0·005$).
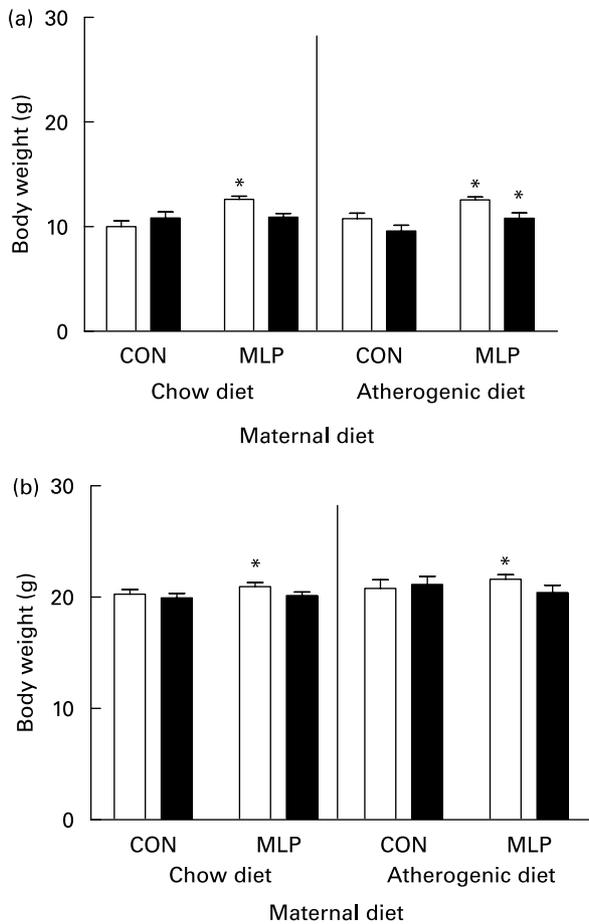
Fig. 1. Body weight at weaning (a) and at cull after 3 months of feeding chow or atherogenic diet (b). CON, maternal control diet; MLP, maternal low-protein diet; (□), female wild-type C57Bl/6J mice; (■), female transgenic ApoE*3-Leiden mice. Data are means, with standard errors represented by vertical bars. For wild-type C57Bl/6J mice: CON chow, n 10; CON atherogenic, n 10; MLP chow, n 11; MLP atherogenic, n 13. For ApoE*3-Leiden mice: CON chow, n 5; CON atherogenic, n 8; MLP chow, n 6; MLP atherogenic, n 6. At weaning there was an effect of maternal diet (P=0·002). At the end of the trial there was an effect of maternal diet (P=0·002). * Mean value was significantly different from CON animals of the same genotype and fed the same postnatal diet (P<0·05).

In ApoE*3-Leiden animals, lesion area was strongly correlated with plasma cholesterol concentration (r 0·791, P<0·001; Pearson's correlation). The effects of both the prenatal protein restriction and the postnatal atherogenic diet were observed only in female offspring. This sex-specificity is a well-established feature of the ApoE*3-Leiden strain resulting from differences between males and females in terms of VLDL production and clearance rates within the liver[12]. Thus, whilst females develop profound hypercholesterolaemia in response to cholesterol in the diet, the males are largely unaffected. Interestingly among humans carrying the same Leiden mutation, dysbetalipoproteinaemia is seen in both sexes[18].

Animals exposed to a low-protein diet *in utero* exhibited dysregulated lipid metabolism resulting in increased levels of circulating plasma lipids. The liver is the major organ in the control of lipid homeostasis, and so, to assess the potential mechanisms that caused the profound hypercholesterolaemia and increased lesion area in the aortic arch, we employed a
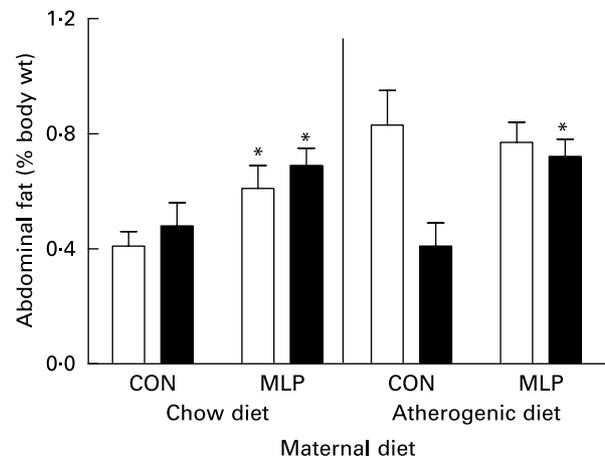
Fig. 2. Total perirenal and gonadal fat depot weight corrected for body weight to provide an indicator of relative fat depot size. CON, maternal control diet; MLP, maternal low-protein diet; (□), female wild-type C57Bl/6J mice; (■), female transgenic ApoE*3-Leiden mice. Data are means, with standard errors represented by vertical bars. For wild-type C57Bl/6J mice: CON chow, n 10; CON atherogenic, n 10; MLP chow, n 11; MLP atherogenic, n 13. For ApoE*3-Leiden mice: CON chow, n 5; CON atherogenic, n 8; MLP chow, n 6; MLP atherogenic, n 6. There was an effect of maternal diet (P=0·023). There was an interaction of maternal and postnatal diets (P=0·002). * Mean value was significantly different from CON animals of the same genotype and fed the same postnatal diet (P<0·05).
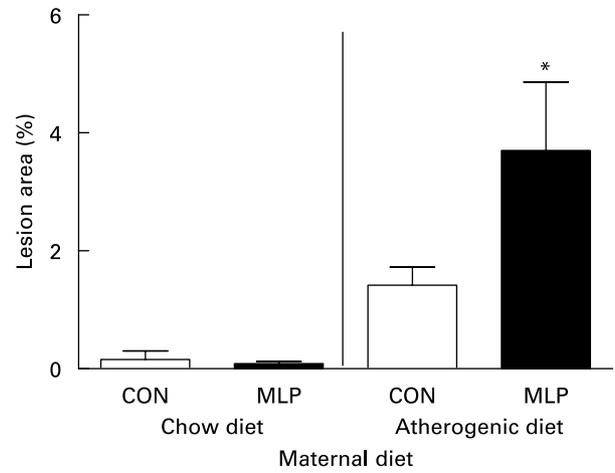
targeted DNA Microarray to analyse any changes in hepatic gene expression (Oligo GEArray® DNA Microarray: Mouse Atherosclerosis, SuperArray). Total RNA isolated from the livers of female ApoE*3-Leiden mice was used to synthesise cRNA, which was hybridised to a pathway-specific microarray profiling the expression of 113 key genes involved in atherosclerosis. Table 3 shows the expression of genes from this array significantly affected by either prenatal or postnatal dietary challenges. Criteria for acceptance as a candidate gene for further investigation were observation of expression above minimum threshold levels, a fold change ≥2, or fold change ≤ 0·5, comparing maternal control and low-protein diets and significance at P<0·05, derived from two-way ANOVA of four to six array measurements per group. Using this fold change approach we identified twelve genes which were significantly regulated by prenatal undernutrition and a postnatal atherogenic diet. Expression of all of these targets was suppressed in low-protein-exposed, compared with maternal control diet-exposed offspring. The majority of gene expression changes were to cytokines, growth factors and their receptors (nine out of twelve genes). The role of these genes in the liver in relation to atherosclerosis is questionable. Suppressed expression of the LDLr and retinoid X receptor in low-protein-exposed livers was of greater interest, given their established roles in the hepatic metabolism of cholesterol and fatty acids[19,20].

Given the observed association between plasma cholesterol concentration and the degree of atherosclerosis noted in the mice, we chose the LDLr as a candidate for further investigation following the microarray results. The LDLr is a cell-surface receptor responsible for the endocytosis of cholesterol-rich LDL. The LDLr recognises apoB100 on LDL particles, apoE on chylomicron remnants and VLDL particles. LDL is directly involved in the pathogenesis of atherosclerosis
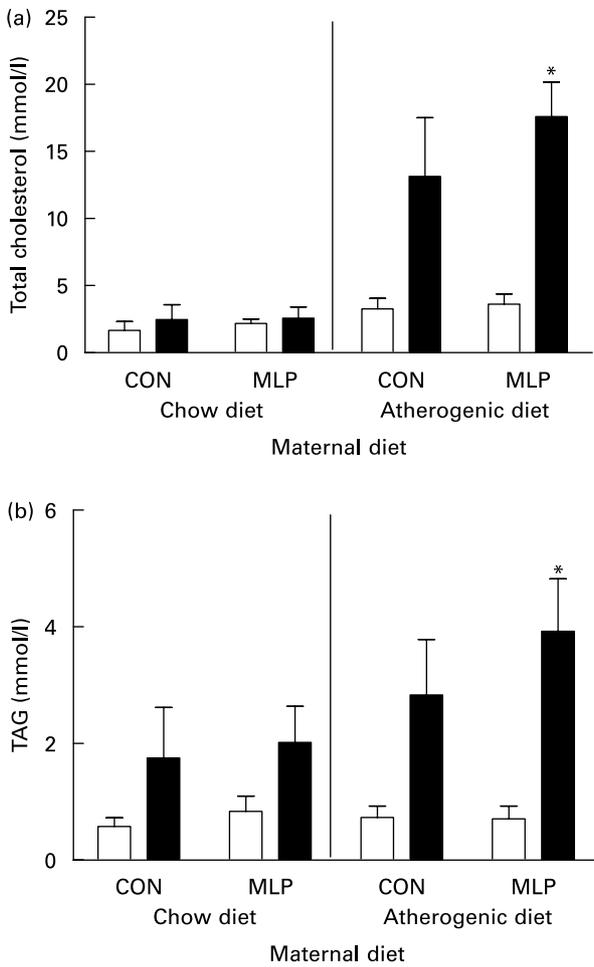
Fig. 3. Cholesterol (a) and TAG (b) concentrations. CON, maternal control diet; MLP, maternal low-protein diet; (□), female wild-type C57Bl/6J mice; (■), female transgenic ApoE*3-Leiden mice. Data are means, with standard errors represented by vertical bars. For wild-type C57Bl/6J mice: CON chow, n 10; CON atherogenic, n 10; MLP chow, n 11; MLP atherogenic, n 13. For ApoE*3-Leiden mice: CON chow, n 5; CON atherogenic, n 8; MLP chow, n 6; MLP atherogenic, n 6. ANOVA indicated significant effects of maternal diet (P<0·001), atherogenic diet (P<0·001) and genotype (P<0·001) and interactions of maternal diet with diet and genotype (P<0·05) on both variables. * Mean value was significantly different from CON animals of the same genotype and fed the same postnatal diet (P<0·05).

Fig. 4. Area of intima exhibiting atherosclerotic lesions in female ApoE*3-Leiden mice. CON, maternal control diet; MLP, maternal low-protein diet. Data are means, with standard errors represented by vertical bars. For ApoE*3-Leiden mice: CON chow, n 5; CON atherogenic, n 8; MLP chow, n 6; MLP atherogenic, n 6. ANOVA indicated significant effects of maternal diet (P=0·001), atherogenic diet (P<0·001) and genotype (P<0·001) and interactions of maternal diet with diet and genotype (P<0·05). * Mean value was significantly different from CON animals fed the same postnatal diet (P<0·05).

due to the accumulation of LDL-cholesterol in the blood[21]. Microarray analysis revealed a significant interactive effect of prenatal protein restriction and the postnatal atherogenic diet on LDLr gene expression (mRNA expression down-regulated by 66 % in low-protein-exposed mice; P=0·032). This result was partly verified by quantitative real-time PCR analysis of LDLr gene expression in the livers of female ApoE*3-Leiden mice (P=0·05; Fig. 5 (a)). Although there was no significant interaction between pre- and postnatal dietary influences observed, there was a trend towards decreased LDLr mRNA expression in the animals with increased atherosclerosis that approached statistical significance (P=0·058). To further elucidate possible mechanisms that could contribute to the elevated plasma cholesterol concentrations, we also investigated changes in mRNA expression of two other members of the LDLr family of lipoprotein receptors, which were not included on the targeted DNA

microarray; LDLr-related protein 1 (LRP-1) and VLDL receptor (VLDLr), as shown in Fig. 5 (a). Both of these latter receptors are important for the metabolism of ApoE-containing, TAG-rich lipoproteins. There was no significant effect of prenatal or postnatal dietary manipulations on VLDLr mRNA expression in the liver. There was, however, a significant interactive effect of protein restriction during pregnancy and postnatal atherogenic diet on expression of liver LRP-1 mRNA (P=0·009), which was reduced in animals exposed to a protein-restricted diet *in utero*, particularly in those fed a chow diet postnatally.

Sterol regulatory element-binding proteins (SREBP) are transcription factors with a pivotal role in the regulation of genes involved in lipid and lipoprotein metabolism[22]. Of the three isoforms expressed, SREBP-1c and SREBP-2 are the predominant forms in liver. SREBP-2 has been shown to regulate the majority of enzymes involved in the cholesterol synthetic pathway[23]. Previous studies suggest that SREBP-2 is a primary regulator of LDLr gene expression[24] and that SREBP1c may be a target for programming by maternal protein restriction[16]. We measured the gene expression of all three SREBP isoforms in the liver. These genes were not present on the DNA microarray (Table 1, Fig. 5 (b)). There was no significant effect of either prenatal protein restriction or postnatal atherogenic diet on either SREBP-1a (data not shown) or SREBP-2 (Fig. 5 (b)). The expression of SREBP1c mRNA in liver was significantly lower in mice exposed to the low-protein diet *in utero* and fed chow in postnatal life, but similar in both groups of offspring fed the atherogenic diet (interaction of pre- and postnatal diet; P=0·005). Quantitative real-time PCR confirmed that there was no significant effect of prenatal or postnatal diets on mouse or human ApoE mRNA expression (data not shown). Importantly, it can be concluded that the increased atherosclerosis noted in ApoE*3-Leiden mice exposed to the low-protein diet *in utero* was not a result of programming of increased expression of the transgene.

Table 3. Microarray analysis of changes in gene expression with maternal protein restriction and atherogenic diet in livers from female ApoE*3-Leiden mice (fold changes)

(Mean values for four to six observations per group)

| Gene name	Symbol	GenBank number	Fold change (MLP v. control) on chow diet	Fold change (MLP v. control) on atherogenic diet	Effect of prenatal diet (P)	Fold change atherogenic v. chow diet	Effect of postnatal diet (P)	Prenatal × postnatal diet interaction (P)
Chemokine (C−C motif) ligand 2	Ccr2	NM_009915	0·31	0·37	0·004	0·67	0·737	0·004
Intracellular adhesion molecule 2	Icam2	NM_010494	0·32	0·53	0·023	0·50	0·630	0·023
IL-10	Il10	NM_010548	0·26	0·34	0·003	0·61	0·036	0·004
IL-1 receptor, type 1	Il1r1	NM_008362	0·31	0·54	0·014	0·63	0·864	0·014
Kinase insert domain protein receptor	Kdr	NM_010612	0·31	0·33	0·005	1·14	0·868	0·005
LDL receptor	Ldlr	NM_010700	0·33	0·34	0·032	0·73	0·807	0·032
Leukaemia inhibitory factor	Lif	NM_008501	0·45	0·24	0·029	0·63	0·589	0·029
Platelet-derived growth factor a	Pdgfa	NM_008808	0·24	0·39	0·045	0·64	0·340	0·045
Retinoid X receptor	Rxra	NM_011305	0·34	0·24	0·027	0·64	0·000	0·027
Superoxide dismutase 1, soluble	Sod1	XM_128337	0·46	0·48	0·018	0·28	0·000	0·018
Transforming growth factor, β 3	Tgfb3	NM_009368	0·41	0·38	0·005	0·80	0·010	0·005
Tumour necrosis factor	Tnf	NM_013693	0·31	0·28	0·007	0·51	0·007	0·007

Discussion

The key finding of the present study is that the feeding of a maternal low-protein diet programmes cholesterol metabolism and/or transport in the female atherosclerosis-prone ApoE*3-Leiden mouse. These metabolic changes appeared to be directly linked to the formation of a greater area of atherosclerotic lesions within the aortic arch. These findings are of major importance, as this is the first demonstration using an animal model that maternal undernutrition, as opposed to overnutrition[5,6], can programme the outcome of CVD, as opposed to simply cardiovascular risk factors. As such the study provides evidence to support Barker's developmental origins of adult disease hypothesis[2].

The mechanisms that link the maternal diet to development of atherosclerosis are, as yet, not well understood. It is clear from the present study that the programming of atherosclerosis and associated changes in lipid profile are specific metabolic and physiological effects of the low-protein diet. There was no impact of the low-protein diet upon litter size, upon the male:female ratio, the wild-type:transgenic mice ratio or postnatal survival. This allows us to rule out effects of protein undernutrition upon intra-uterine or perinatal survival as drivers of later responses to the atherogenic diet. We also noted that the low-protein-exposed mice appeared to have better growth to weaning and were slightly fatter than offspring of mice fed the control diet in pregnancy. In the present study offspring were not weighed at birth in order to avoid disturbing the suckling mothers. Although the study lacks these important data, we would assert that our findings are unlikely to be the result of undernutrition followed by catch-up growth and are instead due to specific mechanisms impacting on lipid metabolism and transport. It has consistently been shown that exposure to moderate protein restriction during pregnancy (9 % by weight) results in low–normal birth weight in rats[10,25,26]. The finding of similar patterns of hepatic gene expression in these mice and in rats[16] following exposure to low-protein diets *in utero* suggests that species differences have little impact upon the programmed responses that follow protein undernutrition in rodent pregnancy.

The present study has attempted to clarify potential mechanisms that result in the increased concentrations of plasma cholesterol and the degree of atherosclerotic injury. Certainly there appears to be fetal programming of cholesterol metabolism, as the low-protein-exposed mice exhibited a greater degree of hypercholesterolaemia in response to the atherogenic diet. Correlations and regression analysis indicated that plasma cholesterol concentrations were directly related to the extent of atherosclerosis ($P < 0.001$). Our microarray studies indicated a potential role for the LDLr, although this was not entirely confirmed by quantitative PCR. Follow-up studies were suggestive of a role for another member of the LDLr family, LRP-1, and transcription factors that regulate lipoprotein metabolism in mediating the greater extent of disease. The LDLr and LRP-1 have well-established hepatic roles in the removal of pro-atherogenic lipoproteins from the plasma[27]. A step-wise linear regression model suggested that although changes in LDLr, LRP-1 and SREBP-1c mRNA expression in the liver were not directly associated
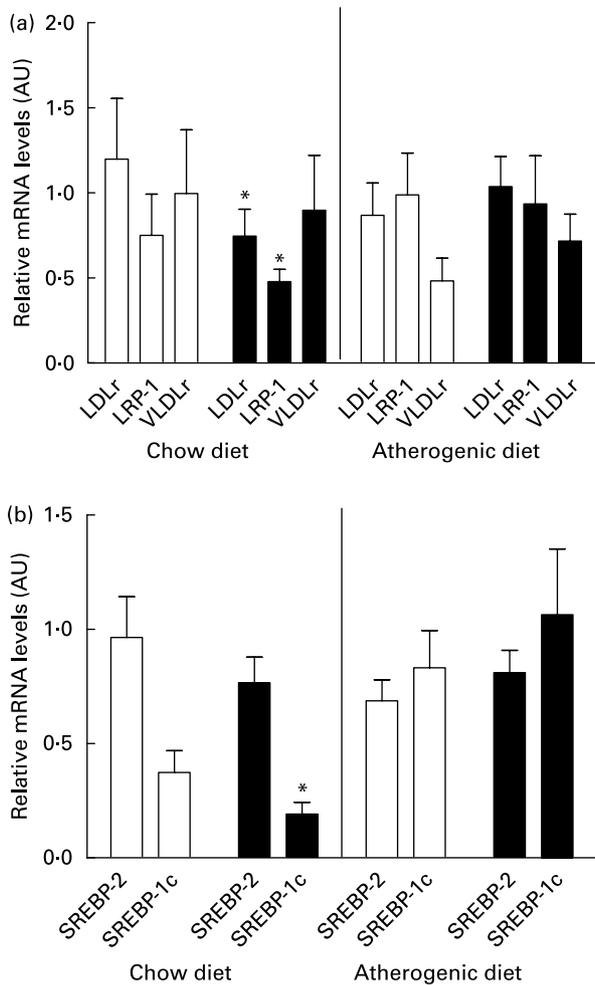
Fig. 5. RT-PCR quantification of hepatic mRNA levels in female ApoE*3-Leiden mice. (a) Relative mRNA levels of liver LDL receptor (LDLr), LDLr-related protein 1 (LRP-1) and VLDL receptor (VLDLr). (b) Relative mRNA levels of liver sterol regulatory element-binding protein (SREBP)-2 and SREBP-1c. (□), Maternal control diet (CON); (■), maternal low-protein diet (MLP); AU, arbitrary units. Data were normalised to the geometric mean of four housekeeping genes and are shown as means, with standard errors represented by vertical bars. For ApoE*3-Leiden mice: CON chow, n 5; CON atherogenic, n 7; MLP chow, n 5; MLP atherogenic, n 6. Mixed-model analysis indicated significant effects of maternal diet on LDLr ($P=0.05$), SREBP-1c ($P=0.009$) and LRP-1 ($P=0.009$), of atherogenic diet on LDLr ($P=0.042$) and SREBP-1c ($P=0.035$) and interactions of maternal diet with atherogenic diet on SREBP-1c ($P=0.005$) and LRP-1 ($P=0.009$). * Mean value was significantly different from CON animals fed the same postnatal diet ($P<0.05$).

with lesion area, LRP-1 expression was significantly related to plasma cholesterol concentrations ($P=0.027$). This suggests that changes in hepatic gene expression in response to maternal protein restriction, particularly that of LRP-1, modulated the circulating levels of cholesterol, which in turn drove the increase in atherosclerosis. Loss of LRP-1 expression in the livers of mice lacking expression of the LDLr leads to the accumulation of cholesterol-rich lipoproteins in the plasma[27]. We noted under-expression of the LDLr and suppression of LRP-1 in the livers of low-protein-exposed mice, which also displayed a hyperlipidaemic plasma (lipid) profile. We hypothesise that the programming effects of the low-protein diet upon hepatic gene expression observed in the chow-fed animals may represent the baseline

phenotype against which the atherogenic diet exerts disease-inducing effects. Whilst mice fed an atherogenic diet postnatally showed few significant differences between control and low-protein-exposed offspring, this is likely to reflect adaptations to 3 months of consuming a diet with a higher fat content. Further studies that consider a time course of responses to the atherogenic diet will be necessary to test this hypothesis, as gene expression measurements were only made at 16 weeks of age, after 12 weeks of feeding the postnatal diets. Given that the mRNA analyses provide only preliminary evidence of likely mechanisms, it would also be desirable to confirm changes in gene expression at the protein level.

We have previously shown in rats that many aspects of lipid metabolism, including expression of SREBP1c, are programmed by fetal protein restriction, and that plasma total cholesterol is elevated in older female offspring[12]. Other studies have suggested that maternal hypercholesterolaemia during pregnancy may programme atherosclerosis in the offspring of rabbits and LDLr knockout mice[5,6,28,29]. These studies provide important information about the mechanisms that link maternal overnutrition to the development of atherosclerosis in the developing fetus. It is unlikely that the under-nutrition experienced during pregnancy by the mice in the present study would programme disease through the same mechanisms, since all mothers used within the study were of the wild-type C57BL/6 background strain which are relatively resistant to the development of lipid abnormalities, even when fed a diet rich in cholesterol and saturated fat (see Fig. 3). It is also unlikely that a mild–moderate restriction of protein against a 10 % maize oil diet containing no cholesterol would impact upon maternal plasma lipid profiles. However, we do acknowledge that one of the limitations of the present study was that no measurements were made of the maternal metabolic profile whilst consuming the low-protein diet. It would be of considerable interest to test whether this diet could modify lipid profiles and mediate an increased risk of atherosclerosis in the offspring through the same mechanism as noted in the rabbit and LDLr models. It should be noted, however, that our experience from studies of pregnant rats fed the same diet is that TAG and total cholesterol concentrations do not change with low protein feeding (S Engeham and SC Langley-Evans, unpublished results).

We have shown that the development of atherosclerosis is dependent on the interaction of genotype, prenatal diet and postnatal diet. This highlights the importance of gene–nutrient interactions at very early stages of life in the aetiology of disease, and indicates that the nature of those interactions influences the responses made to dietary challenges at later stages. This is the first study to demonstrate, experimentally, that undernutrition during fetal life can determine the risk of developing atherosclerosis in adulthood. As such it provides strong support for the developmental origins of health and disease hypothesis[1,7]. It is important to note that within the study we only examined the effects of a single level of protein restriction upon the development of atherosclerosis. Further studies will need to examine whether there is a linear dose–response relationship, or whether as with hypertension in rats subject to protein restriction, there is a simple threshold at which programmed responses occur[10].

The ApoE*3-Leiden mouse is a unique resource in that the postnatal diet rich in cholesterol is an absolute requirement for the appearance of the atherosclerotic phenotype. This mirrors the aetiology of human atherosclerosis and as such makes the ApoE*3-Leiden mouse an ideal model for further explanation of the mechanistic basis of fetal programming. Given that a high proportion of adults in countries currently undergoing economic and nutritional transition will have been exposed to suboptimal nutrition *in utero*, these findings may have major implications for global public health.

Acknowledgements

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