Experimental Physiology

Programmed aortic dysfunction and reduced Na^+,K^+-ATPase activity present in first generation offspring of lard-fed rats does not persist to the second generation

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We have previously reported that male and female offspring of Sprague–Dawley rats fed a diet rich (approximately 50% of caloric intake from fat) in animal fat (lard) during pregnancy and suckling (OHF) demonstrate cardiovascular dysfunction, including blunted endothelium-dependent vasodilatation in the aorta as well as reduced renal Na^+,K^+-ATPase activity. Cardiovascular dysfunction has been reported in other models of developmental programming and some researchers describe transmission from F_1 to F_2 generations. Here we report a study of vascular function, as assessed in isolated rings of aorta mounted in an organ bath, and renal Na^+,K^+-ATPase activity in 6-month-old male and female F_2 offspring of lard-fed and control-fed (OC) dams ($n = 13$ per diet group). An increase in brain (OC 0.61 $\pm$ 0.01% *versus* OHF 0.66 $\pm$ 0.02% of bodyweight) and kidney weights (OC 0.32 $\pm$ 0.01% *versus* OHF 0.37 $\pm$ 0.01% of bodyweight) was observed in female F_2 offspring of lard-fed dams compared with F_2 controls ($P < 0.03$). Constrictor responses to phenylephrine in the aorta were not different from F_2 controls (repeated measures ANOVA, $P = 0.85$). Also, endothelium-dependent dilator function, as assessed by responses to acetylcholine (repeated measures ANOVA, $P = 0.96$) and passive distensibility in the absence of extracellular calcium (repeated measures ANOVA, $P = 0.68$), was similar. Additionally, renal Na^+,K^+-ATPase activity was not statistically different from that observed in control animals (ANOVA, $P = 0.89$). Although a maternal diet rich in animal fat has deleterious effects on parameters of cardiovascular risk in F_1 animals, it does not appear that disorders previously reported in the F_1 generation are transmitted to the F_2 generation.

(Received 5 November 2006; accepted after revision 17 January 2007; first published online 25 January 2007)
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Dietary fat consumption is high in the western world, and in the UK it is estimated that the average person receives 37.6% of total energy from all fats, of which saturated fats account for 14.8% of the calories consumed (Office of National Statistics (UK), 2006).

Whilst excessive dietary fat intake is a recognized risk factor for adult cardiovascular disease, animal studies have suggested that *in utero* exposure to a maternal diet rich in fat may lead to heightened risk of adulthood cardiovascular disease in the offspring (Palinski *et al.* 2001; Armitage *et al.* 2004*b*, 2005*b*; Khan *et al.* 2005; Taylor *et al.* 2005). There is limited evidence from other models of developmental programming that some interventions in pregnancy are transmitted from the first generation offspring (F_1) and thus persist to the next generation (F_2), and even to the third generation. Amongst the earliest evidence was the observation that insulin resistance acquired in F_1 animals by exposure to an *in utero* diabetic environment persists to the F_2 generation (Van Assche & Aerts, 1985; Oh *et al.* 1991). Exogenous glucocorticoid administration to pregnant rats results in abnormal glucose tolerance in F_2, but curiously not the F_1, generation offspring (Drake *et al.* 2005), and maternal protein restriction results in abnormal glucose tolerance in first- and second-generation offspring

 DOI: 10.1113/expphysiol.2006.036202

Table 1. Calculated fatty acid composition of experimental diets

Fatty acid	Control diet	Fat-rich diet
14:0	0.91	1.61
16:0 (palmitic acid)	15.6	25.5
18:0 (stearic acid)	2.61	25.5
20:0	0.22	0.17
22:0	0.14	n.a.
24:0	0.08	n.a.
ΣSFA	19.5	39.4
16:1 (palmitoleic acid)	1.15	2.7
18:1 (oleic acid)	19.0	39.3
20:1	—	0.91
22:1	—	0.20
24:1	0.16	n.a.
ΣMUFA	20.3	41.1
18:2n-6 (linoleic acid, LA)	47.9	15.6
20:2n-6	0.11	0.31
20:3n-6	n.a.	n.a.
20:4n-6 (arachidonic acid, AA)	0.10	0.16
Σn-6 PUFA	48.1	16.1
18:3n-3 (α-linolenic acid, ALA)	5.37	1.58
22:5n-3	0.28	0.30
22:6n-3 (docosahexaenoic acid, DHA)	1.41	0.30
Σn-3 PUFA	7.3	2.18
18:2n-6/18:3n-3	8.92	9.87
n-6/n-3	6.59	7.39

All fatty acids are given as a percentage of total composition, except for ratios. Abbreviations: SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; and PUFA, polyunsaturated fatty acids. Key fatty acids that are referred to throughout the text are named and their abbreviation given. n.a. indicates not assessed.

but only when protein restriction is confined to pregnancy and not lactation in the F_0 dams (Zambrano *et al.* 2005). Endothelial dysfunction acquired in the F_1 generation of animals prenatally exposed to protein deprivation has also been shown to be present in F_2 animals (Brawley *et al.* 2003). There are also isolated reports in man which would suggest that nutrition in grandparents may be a determinant of cardiovascular risk (Kaati *et al.* 2002).

In light of this emerging evidence for intergenerational programming, we examined the effect of a maternal saturated-fat-rich diet on second-generation offspring in the Sprague–Dawley rat. We focused on two parameters that we previously reported to be abnormal in F_1 animals: dilator function in the isolated aorta and the activity of the Na^+,K^+-ATPase (Armitage *et al.* 2004*a*).

Methods

All procedures involving animals were carried out with adherence to guidelines set out by the United Kingdom Animals (Scientific Procedures) Act 1986 and the local animal ethics committee.

Dietary and breeding protocols

Sprague–Dawley rats of breeding age (90–100 days old; Charles River Laboratories, UK) were habituated to the animal facility and maintained under climatically controlled conditions (24°C, 60% humidity, light < 200 Lux, 12 h–12 h light–dark cycle), then assigned either a control diet (Rat and Mouse RM3, Special Diet Services, Witham, UK) or a lard-rich diet comprising the control diet premix supplemented with 20% (w/w) animal lard (Special Diet Services). The composition of these diets has been described in detail previously (Taylor *et al.* 2003). Briefly, the premix control diet comprised 5.3% fat as corn oil, 21.2% protein, 57.4% carbohydrate and 4.6% fibre. The lard-rich diet was made up from the control diet, with addition of animal lard (20% w/w), leading to a final composition of 25.7% fat, 19.5% protein, 41.3% carbohydrate and 3.5% fibre. The fatty acid composition of these experimental diets is given in Table 1. The lard-rich diet provides a fatty acid profile component that is comparable to that of a 'fast food' western diet, being high in saturated and mono-unsaturated fatty acids but low in essential omega-3 polyunsaturated fatty acids. The control diet is primarily comprised of essential omega-3 and 6 polyunsaturated fatty acids, theoretically providing beneficial fatty acid intake. Extra vitamin and mineral premix was added to the lard-rich diet to ensure that the addition of lard did not reduce the micronutrient content. A total of 20 ($n = 10$ per diet group) female Sprague–Dawley rats (F_0 animals) were fed one of these diets for 10 days prior to mating, during pregnancy and throughout the suckling period. Within 48 h of birth, offspring (F_1) litters were standardized to 8 pups with equal numbers of males and females and weaned onto the standard chow diet (RM3 Special Diet Services) at 21 days of age. The F_1 females ($n = 10$ per F_0 diet group) were mated at 90–110 days of age and remained on the standard diet throughout pregnancy and suckling. Their offspring (F_2 generation) were subjected to the same litter standardization procedure as F_1 and remained on the standard diet throughout life. No more than one female from each of the F_0 and F_1 litters were used for breeding to avoid intralitter bias. Figure 1 shows the breeding protocol.

At 6 months of age, F_2 animals ($n = 13$ per F_0 diet group, comprising $n = 6$–7 male and female offspring) were killed by cervical dislocation. Aortas and kidneys were rapidly dissected in ice-cold physiological saline solution (PSS) and used for study of aortic function or Na^+,K^+-ATPase activity, respectively. Bodyweight was recorded, and visceral fat pad, liver, heart, lung, spleen, brain and kidney were dissected, blotted dry and weighed. All methodologies were the same as employed in a previous study of aortic and Na^+,K^+-ATPase function (Armitage *et al.* 2005*a*).

Aortic function

Isolated thoracic aorta (comprising the section of aorta from the end of the aortic arch to the diaphragm) was dissected free of connective tissue and fat. The same length of aorta was collected from all animals, and use of the middle portion of the collected tissue ensured that all aortic rings used originated from the same area of thoracic aorta. Two rings, 2.5 mm in length from the middle portion of each vessel, were mounted in an organ bath (Model 700MO, DanishMyo, Aarhus, Denmark) containing physiological salt solution (PSS composition (mM); Nacl (119), KCL (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.17), $NaHCO_3$ (25), NaH_2PO_4 (1.18), EDTA (0.026), glucose (6.0)) at 37°C, gassed with 95% O_2–5% CO_2 and equilibrated to a 5 mN force (equivalent to 1 g) for 30 min. Vessels were then subjected to a run-up of three cumulative stretches (from basal internal diameter, stretched by 500, 200 and then 50 μm), each lasting 2 min, to construct a circumference–tension curve. After re-equilibration at 5 mN, three contractile responses to 125 mM K^+-substituted PSS (KPSS) were conducted. Vessels were then washed and equilibrated at a force of 5 mN and a cumulative dose–response curve performed with phenylephrine (3×10^{-9} to 10^{-5} M). After washout, vessels were submaximally constricted with phenylephrine (80% of maximum force), then dose–response curves constructed to assess endothelium-dependent vasodilatation following application of acetylcholine (ACh, 3×10^{-9} to 10^{-5} M). Smooth muscle sensitivity to the endothelium-independent vasodilator nitric oxide was assessed by a dose–response curve using aqueous nitric oxide solutions (10^{-7} to 3×10^{-5} M). The PSS was then substituted with warmed calcium-free PSS (with 10^{-4} M EGTA) and the passive circumference–force relationship (a measure of passive stiffness) repeated over three cumulative stretches (500, 200 and 50 μm), each lasting 2 min. Two rings from each aorta were studied and data averaged in subsequent analyses.

Na^+,K^+-ATPase activity assay

The assay of Na^+,K^+-ATPase activity is based on measurement of organic phosphates produced when the Na^+,K^+-ATPase hydrolyses ATP to ADP (Else *et al.* 1996). By comparing phosphate release in tissue homogenates with and without addition of the Na^+,K^+-ATPase inhibitor ouabain, Na^+,K^+-ATPase specific phosphate generation is estimated. Full methodology and protocol have been previously reported (Armitage *et al.* 2005*a*). Briefly, small fragments of renal cortex were homogenized in a sucrose-based buffer, cell membranes disrupted by the addition of SDS and then the homogenate incubated in a solution containing sodium and potassium ouabain (10 mM). After equilibration at 37°C, excess ATP was added for 5 min and the reaction quenched with phosphoric acid. Phosphate liberation was measured by colorimetric reaction on an automated plate reader and activity expressed as moles phosphate liberated per hour per milligram protein.

Results

Allometry

Table 2 gives the body and organ weights of F_2 rats from lard- and control-fed dams. Organ weights were expressed as a percentage of bodyweight. There was a significant

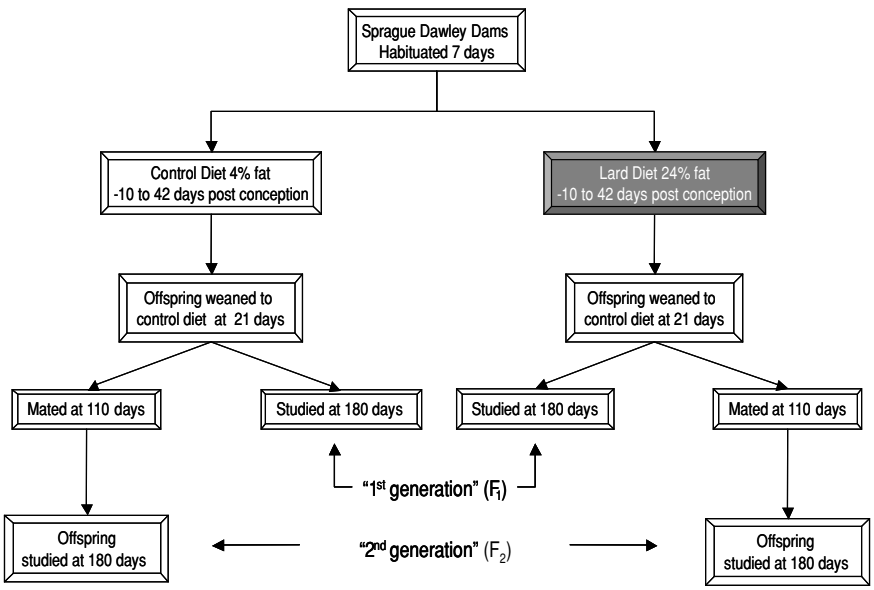

Figure 1. Schematic diagram of the breeding and dietary protocols used to produce F_2 offspring of lard-fed F_0 dams

Table 2. Body and organ weights in male and female F_2 offspring

	Control diet		Fat-rich diet		Significance	
Tissue	Male ($n = 7$)	Female ($n = 7$)	Male ($n = 6$)	Female ($n = 6$)	Sex	Diet
Bodyweight (g)	469.7 ± 14.8	274.4 ± 9.4	500.1 ± 26.5	284.7 ± 8.2	< 0.0001	0.49
Heart (% body wt)	0.34 ± 0.02	0.37 ± 0.01	0.32 ± 0.02	0.37 ± 0.01	0.02	0.58
Liver (% body wt)	4.2 ± 0.8	3.5 ± 0.2	3.8 ± 0.1	3.7 ± 0.2	0.006	0.79
Spleen (% body wt)	0.20 ± 0.01	0.24 ± 0.01	0.24 ± 0.05	0.23 ± 0.03	0.36	0.46
Kidney (% body wt)	0.31 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.37 ± 0.01	0.0003	0.03
Brain (% body wt)	0.40 ± 0.02	0.61 ± 0.01	0.41 ± 0.02	0.66 ± 0.02	< 0.0001	0.03
Lung (% body wt)	0.39 ± 0.02	0.51 ± 0.02	0.36 ± 0.01	0.49 ± 0.03	< 0.0001	0.30
Fat pads (% body wt)	190 ± 0.26	2.27 ± 0.17	2.36 ± 0.34	2.81 ± 0.39	0.15	0.08

Data are given as means ± S.E.M. and statistical comparisons are made by two-way ANOVA with F_2 sex and F_0 diet as independent variables. All organ weights are given as a percentage of bodyweight.

effect of sex on organ weight. Kidney ($P < 0.03$) and brain ($P < 0.03$) weight were increased in F_2 female offspring derived from fat-fed F_0 dams compared with F_2 offspring derived from control-fed F_0 dams.

Aortic function

Figure 2 shows the effect of F_0 lard intake on F_2 aortic function for $n = 7$ male and female control and $n = 6$ male and female F_2 offspring of lard-fed dams.

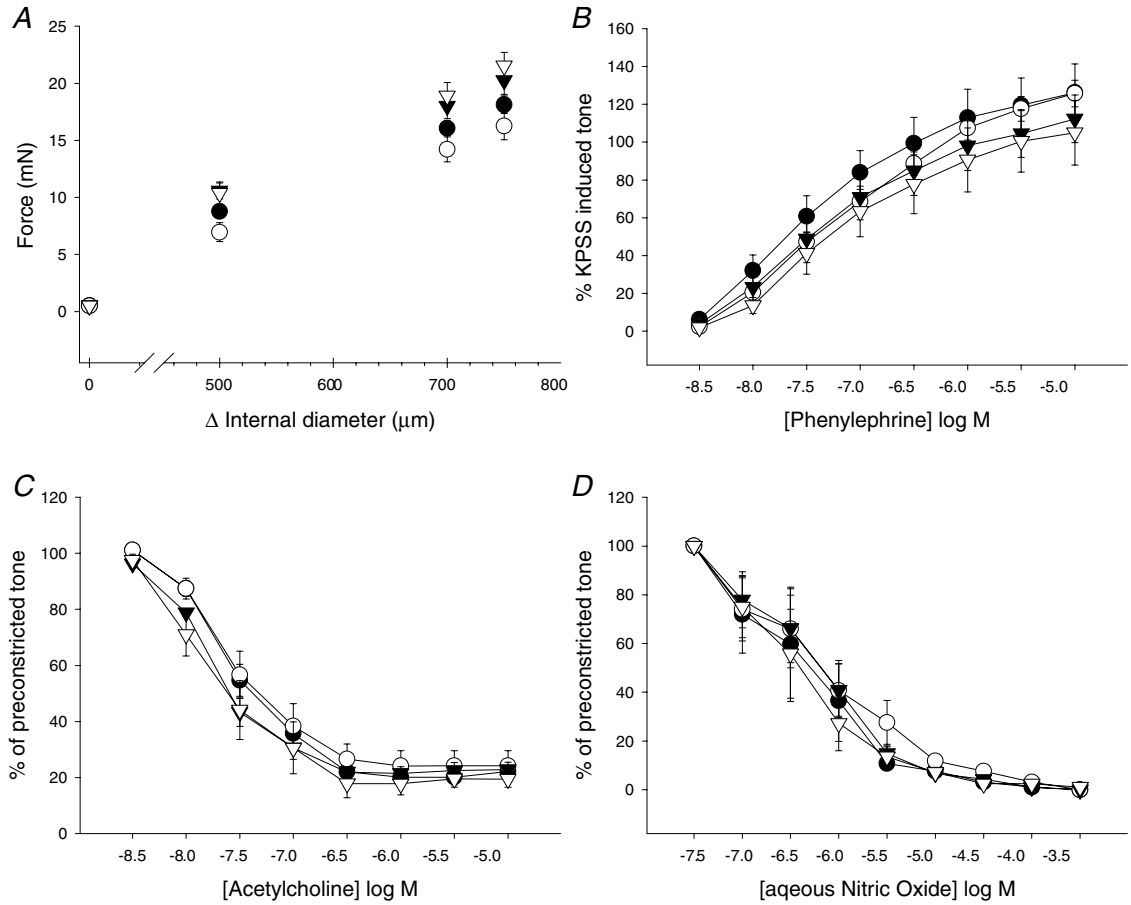

Figure 2. Aortic function in F_2 offspring is not affected by F_0 diet during pregnancy and suckling
In all panels, data represent means ± S.E.M. for $n = 7$ F_2 control male (○) and female (▽) and $n = 6$ male (●) and female (▼) F_2 offspring of lard-fed dams. *A*, there is no effect of F_2 sex or F_0 diet upon passive distensibility. *B*, there is no effect of F_2 sex or F_0 diet upon active constriction in reponse to phenylephrine normalized to the maximal contraction in response to KPSS. *C*, there is no effect of F_2 sex or F_0 diet upon endothelium-dependent vasodilatation in response to ACh. *D*, there is no effect of F_2 sex or F_0 diet upon endothelium-independent vasodilatation in response to aqueous nitric oxide.

There was no transgenerational programming of the aortic circumference–force relationship, with arteries of F_2 offspring derived from lard- and control-fed F_0 dams demonstrating similar force generation over the circumference–force curve in the absence of calcium (Fig. 2*A*, repeated measures ANOVA, diet × stretch, $F_{1,3} = 0.49$, $P = 0.68$). There was, however, a significant effect of sex on the circumference–force relationship, with females showing less elasticity than males (Fig. 2*A*, repeated measures ANOVA, sex × stretch, $F_{1,3} = 7.9$, $P = 0.0002$).

There was no effect of F_0 diet during pregnancy and suckling on F_2 generation aortic contractility in response to phenylephrine (Fig. 2*B*, repeated measures ANOVA, diet × dose, $F_{1,7} = 0.48$, $P = 0.85$), nor was sex of offspring a significant predicator of reactivity to phenylephrine (Fig. 2*B*, repeated measures ANOVA, sex × dose, $F_{1,7} = 0.99$, $P = 0.44$).

Endothelium-dependent dilatation in response to acetylcholine in the F_2 generation was not altered by the diet of the F_0 generation during pregnancy (Fig. 2*C*, repeated measures ANOVA, diet × dose, $F_{1,7} = 0.26$, $P = 0.96$), nor was there an effect of sex of offspring on acetylcholine-stimulated vasodilatation (Fig. 2*C*, repeated measures ANOVA, sex × dose, $F_{1,7} = 1.56$, $P = 0.16$).

Furthermore, the dose–response curves to aqueous nitric oxide were unrelated to F_0 diet (Fig. 2*D*, repeated measures ANOVA, diet × dose, $F_{1,7} = 0.22$, $P = 0.98$) or sex of offspring (Fig. 2*D*, repeated measures ANOVA, sex × dose, $F_{1,7} = 0.14$, $P = 0.99$). Consistent with analyses of the entire dose–response curves, there were no significant effects of F_0 diet during pregnancy and suckling on the computed EC_{50} values for phenylephrine, acetylcholine or aqueous nitric oxide solution in F_2 offspring (data not shown).

Na^+,K^+-ATPase activity

There was no effect of F_0 lard intake during pregnancy and suckling on Na^+,K^+-ATPase activity in kidney homogenates from F_2 offspring (μmol PO_4 liberated $h^{-1} \cdot$ mg protein^{-1}): $n = 6$ control male 175.45 ± 47.3 and female 195.60 ± 15.4; $n = 6$ offspring of lard-fed F_0 dam, male 168.47 ± 47.3 and female 194.5 ± 17.5). There was no significant effect of F_0 lard intake (ANOVA, $F_{1,1} = 0.019$, $P = 0.89$) or F_2 sex (ANOVA, $F_{1,1} = 0.64$, $P = 0.43$) on Na^+,K^+-ATPase activity.

Discussion

We have previously shown that the (F_1) offspring of rats fed a lard-rich diet during pregnancy and suckling show blunted endothelium-dependent vasodilatation in response to ACh and reduced elasticity of the thoracic aorta when compared with offspring of dams fed a normal low-fat chow diet (Armitage *et al.* 2005*a*). We also reported a substantial reduction in activity of Na^+,K^+-ATPase in whole kidney homogenates (Armitage *et al.* 2005*a*). The present study demonstrates that these programmed changes are not transferred through F_1 females to the second (F_2) generation. Our findings of increased brain and kidney weight in female F_2 offspring are consistent with previous reports of sex specificity in intergenerational programming models; however, we cannot attribute any functional significance to these changes in allometry. This observation that programmed functional deficits are present in the F_1 generation but absent in the F_2 generation suggests either that expression of the genes responsible for the programmed F_1 phenotype were not prone to intergenerational epigenetic alteration or that the provision of fat during the F_0 pregnancy induced morphometric changes in F_1 offspring that do not (and would not be expected to) persist to subsequent generations.

We propose that the aortic and renal phenotype we reported in F_1 offspring (Armitage *et al.* 2005*a*) may have occurred at least in part as a result of morphometric changes. For instance, vascular smooth muscle and elastin are laid down early during development (Jacob *et al.* 2001) and may therefore have been prone to alteration by a lipid-rich environment experienced by F_1 offspring. The F_2 generation, however, underwent angiogenesis and vascular development in an environment free from excess dietary saturated fatty acids and thus would not be expected to demonstrate altered vascular function in adulthood. We have previously reported reduced mitochondrial gene expression in the aortas of F_1 offspring of lard-fed dams (Taylor *et al.* 2005). Since mitochondria are inherited through the female lineage, we may expect that any changes in mitochondrial copy number would be transmitted to the F_2 generation. Again, it may be that both an alteration in the mitochondrial genome and an abnormal lipid environment are required to produce a disease phenotype. The activity and expression of various subunit isoforms of the Na^+,K^+-ATPase are also known to change during development (Corthesy-Theulaz *et al.* 1990). Na^+,K^+-ATPase activity is observed in the trophectoderm in the early blastocyst stage (Kidder, 2002), and there is a clear change in predominating Na^+,K^+-ATPase isoforms in rat kidney between conception and 40 days of life (Orlowski & Lingrel, 1988). Na^+,K^+-ATPase activity is also influenced by the local environment, especially the phospholipid composition of the cellular phospholipid membrane (Wu *et al.* 2001).

It is recognized that maternal dietary fat intake can alter offspring membrane fatty acids through altered fatty acid desaturation and elongation (Li *et al.* 2006). Indeed, we have previously demonstrated that the phospholipid membrane composition is altered in F_1

offspring of fat-fed dams (Ghosh *et al.* 2001); therefore, alteration of phospholipid membrane composition may provide an explanation for our observation that F_1 but not F_2 offspring of fat-fed dams demonstrate reduced Na^+,K^+-ATPase activity. The F_2 offspring develop under control dietary conditions with a normal membrane phospholipid environment, and Na^+,K^+-ATPase would therefore be expected to be normal in F_2 animals, as was shown.

The first reports of transgenerational programming of disease came from experimental rat models, whereby exposure to maternal diabetes (induced by streptozotocin) during gestation resulted in macrosomic (F_1) offspring that became hyperglycaemic during pregnancy, giving rise to a macrosomic F_2 generation (Van Assche & Aerts, 1985; Oh *et al.* 1991). Closer examination of later reports of intergenerational programming suggests that very specific conditions might be required to elicit a transgenerational deficit. For example, Zambrano *et al.* (2005) show that intergenerational programming of insulin resistance occurs in F_2 males when the F_1 female was exposed to a control diet (20% protein) during the *in utero* period, followed by a reduced protein diet (10%) during weaning. The F_2 females born to F_1 mothers that were protein restricted *in utero* but then suckled on a control protein diet did not show altered insulin resistance compared with control animals (Zambrano *et al.* 2005). This sex-specific transgenerational programming is therefore dependent on sex of the offspring and a change in protein availability to the mothers during the *in utero* and suckling periods.

Drake *et al.* (2005) administered dexamethasone to F_0 mothers during the last trimester of pregnancy and, although they found no effect of this administration on the F_1 generation, they did observe programming of hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity in F_2 males. Moreover, if the sire of the F_2 male had been exposed to dexamethasone during gestation, this also affected the F_2 male PEPCK activity (Drake *et al.* 2005). This study is unique in that the F_1 generation did not seem affected by exposure to dexamethasone when they were *in utero* but their offspring were affected. This again suggests that only a very specific set of circumstances will lead to developmental programming phenotypes extending through the generations.

To conclude, this study fails to show evidence for intergenerational transmission to the F_2 generation of programming deficits in Na^+,K^+-ATPase activity and endothelium-dependent vasodilatation in the F_1 generation by exposure to a lard-rich diet *in utero* and during the suckling period. Nonetheless, we did observe increases in brain and kidney weight in F_2 offspring of fat-fed dams. Although the increased organ weight was not associated with any overt disease, we cannot rule out the possibility of a more subtle pathology in such animals. Overall, however, when combined with our previous study on F_1 offspring, these data suggest that exposure to a fat-rich diet during gestation and suckling does not necessarily condemn the ensuing genetic line to disease risk. Studies of F_2 generations in the many different models of developmental programming in different species may provide valuable insight into the mechanisms of developmental programming and determine whether transgenerational programming is likely to be of any relevance to disease in man.

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Acknowledgements

This work was funded by the Guy's and St Thomas' Charitable Foundation and the British Heart Foundation. Professor Lucilla Poston is funded by Tommy's, the Baby Charity.