

Increased systolic blood pressure in rats induced by a maternal low-protein diet is reversed by dietary supplementation with glycine

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A B S T R A C T

When rat dams consume a diet low in protein during pregnancy, their offspring develop high blood pressure. On a low-protein diet, the endogenous formation of the amino acid glycine is thought to become constrained. Glycine may become conditionally essential, as its rate of endogenous formation is inadequate to meet metabolic needs, and may be limiting for the normal development of the fetus. In the present study, five groups of Wistar rats were provided during pregnancy with one of five diets: a control diet containing 18% (w/w) casein (CON), a low-protein diet containing 9% casein (MLP), or the low-protein diet supplemented with 3% glycine (MLPG), alanine (MLPA) or urea (MLPU). The offspring were weaned on to standard laboratory chow, and blood pressure was measured at 4 weeks of age. Blood pressure was significantly increased in the MLP, MLPA and MLPU groups compared with the CON group, but for the MLPG group blood pressure was not significantly different from CON. Compared with the CON group, body weight was significantly reduced for the MLP, MLPA and MLPG groups, but for the MLPU group body weight was not different from CON. These data show that different forms of non-essential dietary nitrogen, when consumed during pregnancy, exert different effects upon the growth and function of the offspring. The availability of glycine appears to be of critical importance for normal cardiovascular development.

INTRODUCTION

The nutritional status of a mother and the nutrient environment she provides to the developing fetus determines the extent to which the fetal demand for energy and specific nutrients might be satisfied [1,2]. There is a

body of epidemiological evidence that associates shape and size at birth among different populations with the risk in later life of developing chronic disease, such as cardiovascular disease, Type II diabetes and some cancers [3]. In a systematic review of 80 studies which included 444 000 subjects aged from 0 to 84 years and of all races,

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Abbreviations: EAA, essential amino acid(s); NEAA, non-essential amino acid(s); dietary groups are defined as follows: CON, control diet containing 18% (w/w) casein; MLP, low-protein diet containing 9% (w/w) casein; MLPG, MLPA and MLPU, low-protein diet (9% casein) supplemented with 3% glycine, alanine or urea respectively.

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smaller size at birth was associated with an increase in blood pressure and more prevalent hypertensive disease during adult life [4]. These associations are graded, and are seen across the normal range of birth weights in populations where the infants and children are otherwise seen as normal. In order to determine whether these associations might be causal, animal studies have been carried out in which modest manipulations to the maternal diet during pregnancy have been shown to exert effects upon the metabolism and function of the offspring during later life [5]. The demonstration that variations in dietary composition during pregnancy within the normal range of consumption can have widespread, but specific, effects on the growth and development of the offspring has provided a measure of biological plausibility for the phenomenon of metabolic programming, and provided some evidence for the mechanisms that might underlie the changes observed [6–8].

We have shown that when pregnant rats were provided with modest, graded reductions in dietary protein during pregnancy, the offspring developed higher systolic blood pressure from 4 weeks of age, which remained higher than normal over the entire lifespan [6]. The effect was seen following only 4 days' exposure to the low-protein diet [9], and appeared to be mediated in part through resetting of the hypothalamic–pituitary–adrenal axis and through developmental changes in the structure and function of the kidneys, heart and great vessels [10–13]. However, as the hypertensive effect is not necessarily produced by all diets low in protein [14], this implies that one or more aspects of the low-protein diet provided to the mother during pregnancy limits normal fetal development. There has been a tendency to attribute any effects related to the consumption of low-protein diets to a limitation in the availability of one or more of the essential amino acids (EAA), such as methionine, threonine or lysine [15]. However, the evidence indicates that, as the protein content of the diet is reduced progressively, the availability of total nitrogen, or the ability to maintain adequate formation of specific non-essential amino acids (NEAA), is likely to limit normal growth and function [7,16]. During fetal and neonatal life, the demands for glycine for growth are substantial and its availability appears limited, indicating that the capacity for its *de novo* formation is not adequate to meet demands [17]. While fetal demands for glycine are high, they compete with increased needs in the mother for the formation of haem for the expansion of the red cell pool and other pathways. Use of 5-L-oxoproline as a marker for glycine status has indicated a limitation on its availability during pregnancy and in the newborn [18–20].

Metabolic adaptations occur in response to the consumption of diets low in protein, and successful adaptation requires that the ability to form adequate amounts of NEAA to meet the needs of the body is

maintained [16]. On lower-protein diets there is enhanced retention of urea-nitrogen within the body through the metabolic activity of the colonic microflora, which increases the nitrogen available for the formation of NEAA [21–23]. In rats, the practice of refection means that both EAA and NEAA formed by the colonic microflora from salvaged urea-nitrogen are potentially available to enhance dietary quality for the animal [24]. Normal adults appear to be progressively less able to synthesize adequate amounts of glycine as the level of dietary protein falls, but this limitation can be overcome if additional nitrogen is provided in the diet by compounds as simple as urea [22].

There has been the presumption that, in this rodent model, the provision to the mother of a diet low in protein acts to programme higher blood pressure in the offspring because of inadequacy in the provision of EAA by the diet [15]. There are, however, good reasons for considering that the important difference is an inability to form adequate amounts of NEAA, because the content of non-essential nitrogen in the diet might limit maternal, placental and fetal function, rather than a limitation in any specific EAA [2,7]. In the present study, pregnant female rats were provided with diets in which the protein content was adequate [18% (w/w) casein] or marginal [9% (w/w) casein]. Non-essential nitrogen was added to the 9% casein diet for further groups, as alanine, glycine or urea, so that the nitrogen content of the diet was equivalent to a diet containing 12% (w/w) casein, and the development of the offspring was followed. The hypothesis under test was that the low-protein diet was limiting in glycine, and that the addition of glycine to this diet would have a beneficial effect on blood pressure that would not be seen when alanine or urea was added.

METHODS

A total of 29 virgin female Wistar rats were mated and allocated to one of five experimental diets, which they consumed *ad libitum*. Of these, 26 completed pregnancy successfully. Successful pregnancies resulted in six of seven rats offered an 18% (w/w) casein control diet (CON) [6], six rats offered a low-protein diet (9% casein; MLP), four of six rats offered 9% casein with added urea (MLPU), five rats offered 9% casein with added alanine (MLPA), and five rats offered 9% casein with added glycine (MLPG). All animals had free access to water. Within 12 h of giving birth, the mothers were transferred to a diet of standard laboratory chow (20% protein), which they consumed throughout the suckling period. The five experimental diets were made from raw ingredients, as shown in Table 1. The MLPA, MLPG and MLPU diets had a similar nitrogen content, which increased the nitrogen from that equivalent to a 9% casein diet to that equivalent to a 12% casein diet. The

Table 1 Composition of the experimental diets provided for rats for the duration of pregnancy

Constituent	Diet ... 'Protein' content (w/w) ...	Composition (w/w)				
		CON 18%	MLP 9%	MLPU 12%	MLPA 12%	MLPG 12%
Casein		18	9	9	9	9
Methionine		0.5	0.5	0.5	0.5	0.5
Urea		—	—	1.5	—	—
Alanine		—	—	—	3	—
Glycine		—	—	—	—	3
Snowflake		42.5	48.5	47.5	46.5	46.5
Solkafloc		5	5	5	5	5
Sucrose		21.3	24.3	23.7	23.2	23.2
Choline		0.2	0.2	0.2	0.2	0.2
Mineral mix (AIN-76)		2	2	2	2	2
Vitamin mix (AIN-76)		0.5	0.5	0.5	0.5	0.5
Corn oil		10	10	10	10	10

small differences in macronutrient composition were achieved by adjusting the proportion of carbohydrate in the diets, to ensure that the diets were iso-energetic.

At birth, the litters were culled to eight in each litter for the duration of lactation, and the offspring were weaned on to standard laboratory chow. Systolic blood pressure and pulse were determined at 4 weeks of age in a total of 171 offspring (84 males and 87 females) using a tail cuff system (Blood Pressure Monitor; Linton Instrumentation, Diss, Norfolk, U.K.). Rats were placed in a darkened room maintained at 27 °C for 2 h and settled in a Perspex tube. A suitably sized cuff was placed over the tail and inflated to 300 mmHg, and pulses were recorded during deflation at a rate of 3 mmHg/s. Blood pressure was determined in triplicate for each animal, with the average systolic blood pressure being recorded [9]. Previous studies have shown that this method is reproducible, and gives values similar to those obtained by direct measurement under anaesthesia [25,26]. Animals were killed using an overdose of sodium pentobarbitone, and the brain, liver, lung, spleen, heart and both kidneys were rapidly removed, weighed and frozen in liquid nitrogen.

All statistical analyses were carried out using the SPSS/PC statistical package (10.0). Comparisons between groups were carried out using a general linear model, with diet and sex as fixed variables, with the independent effect of each and any interaction being sought. Body weight was entered as a covariate where appropriate. *Post hoc* comparisons were made using the Bonferroni correction. Differences were accepted as statistically significant at the 5% level. Using individual pups as the cases, there were significant effects of body weight, sex and dietary group on blood pressure, and therefore the analysis was repeated using the average value for each litter as the dependent variable of relevance.

RESULTS

There was no significant difference in the average number of pups in the litters for the different dietary groups: CON, 8; MLP, 8; MLPU, 9; MLPA, 10; MLPG, 10. The body weights of male and female offspring at 4 weeks of age are shown in Table 2. There was a highly significant difference in body weight by gender, with males being about 10% heavier ($P < 0.001$), and by dietary group ($P < 0.001$), but there was no interaction between the two. The mean body weight of animals in the CON and MLPU groups was not different, and both groups were significantly heavier than the MLP, MLPA and MLPG groups, by up to 20%. The weights of the brain, liver, heart, kidneys, lungs and spleen are shown in Table 3. As body weight was entered as a covariate, the values are adjusted to a body weight of 73 g. Using this analysis, there was no difference in tissue mass in relation to sex (other than for the spleen), and there was no interaction between sex and dietary group. Diet had a significant effect upon the relative weights of brain, liver, lung and spleen. The relative brain weight was greatest in the MLPA and MLPU groups, and least in the CON and MLP groups. For liver, the relative weight was least in CON, greatest in MLP and intermediate in the other groups. By contrast, relative lung weight was greatest in MLP and least in MLPA, with the other groups being intermediate. The differences in relative kidney weight failed to achieve statistical significance (diet effect: $P = 0.054$), but there was a trend towards an increase in the MLP, MLPU and MLPA groups compared with CON and MLPG. For the spleen there were highly significant effects of both sex and diet, but no interaction between the two. Relative spleen weight was greater in males than in females. Those animals given the MLP diet had the greatest relative spleen weight; that of the MLPA group

Table 2 Body weight, blood pressure and pulse at 4 weeks of age in male and female offspring of rats given one of five experimental diets for the duration of pregnancy

Values are means \pm S.E.M. Differences among dietary groups were sought using a general linear model in which diet and sex were entered as fixed factors, and body weight was entered as a covariate. Differences among dietary groups based upon litter means were sought using one-way ANOVA. Groups that share the same superscript are not significantly different from each other. NS, not significant.

Parameter	CON	MLP	MLPU	MLPA	MLPG	P value			
						Sex	Diet	Sex* diet	Body weight
Number (male/female)	14/20	21/20	15/15	18/16	16/16				
Body weight (g)									
Male	84.8 \pm 2.8	68.8 \pm 2.3	88.0 \pm 2.7	75.7 \pm 2.5	72.4 \pm 2.6	< 0.001	< 0.001	NS	
Female	78.8 \pm 2.6 ^a	61.6 \pm 2.4 ^b	65.2 \pm 2.7 ^a	68.6 \pm 2.6 ^b	64.8 \pm 2.6 ^b				
By litter mean	87.9 \pm 4.7 ^a	65.7 \pm 4.7 ^b	81.0 \pm 5.7 ^{a,b}	72.2 \pm 5.1 ^{a,b}	69.1 \pm 5.7 ^b	0.026			
Blood pressure (mmHg)									
Male	103.7 \pm 4.8	122.5 \pm 3.7	114.7 \pm 4.8	113.3 \pm 4.0	99.1 \pm 4.2	0.006	< 0.001	NS	0.023
Female	109.0 \pm 4.2 ^a	122.9 \pm 4.1 ^b	128.5 \pm 4.4 ^b	127.6 \pm 4.3 ^b	104.5 \pm 4.4 ^a				
By litter mean	110.5 \pm 4.3 ^{a,b}	121.9 \pm 7.6 ^a	124.5 \pm 3.1 ^a	119.7 \pm 4.6 ^a	100.2 \pm 2.8 ^b		0.045		
Pulse (beats/min)	401 \pm 7.3 ^a	395 \pm 6.4 ^a	388 \pm 7.4 ^a	350 \pm 6.6 ^b	382 \pm 6.9 ^a	NS	< 0.001	NS	NS

Table 3 Organ weight at 4 weeks of age in male and female offspring of rats given one of five experimental diets for the duration of pregnancy

Values are means \pm S.E.M. Differences between groups were sought using a general linear model in which diet and sex were entered as fixed factors, and body weight was entered as a covariate. Groups that share the same superscript are not significantly different from each other. NS, not significant.

Organ	CON	MLP	MLPU	MLPA	MLPG	P value			
						Sex	Diet	Sex* diet	Body weight
Brain (g)	1.46 \pm 0.013 ^a	1.48 \pm 0.011 ^{a,b}	1.52 \pm 0.01 ^{b,c}	1.53 \pm 0.009 ^c	1.51 \pm 0.01 ^{a,b,c}	NS	< 0.002	NS	< 0.001
Liver (g)	3.41 \pm 0.13 ^a	4.05 \pm 0.12 ^b	3.59 \pm 0.13 ^{a,b}	3.68 \pm 0.13 ^{a,b}	3.70 \pm 0.13 ^{a,b}	NS	0.016	NS	< 0.001
Heart (mg)	435 \pm 15	469 \pm 13	454 \pm 15	438 \pm 13	428 \pm 14	NS	NS	NS	< 0.001
Kidneys (mg)	720 \pm 22	788 \pm 20	786 \pm 22	774 \pm 20	728 \pm 22	NS	NS	NS	< 0.001
Lungs (mg)	604 \pm 25 ^{a,b}	681 \pm 22 ^a	599 \pm 25 ^{a,b}	585 \pm 23 ^b	598 \pm 24 ^{a,b}	NS	0.022	NS	< 0.001
Spleen (mg)									
Male	290 \pm 12	296 \pm 9	282 \pm 10	261 \pm 8	300 \pm 9	< 0.001	< 0.001	NS	< 0.001
Female	255 \pm 10 ^{a,b}	290 \pm 10 ^a	245 \pm 9 ^{a,b}	249 \pm 9 ^b	275 \pm 9 ^a				

was least, and the CON, MLPU and MLPG groups were intermediate.

Pulse and blood pressure at 4 weeks of age are shown in Table 2. There was a significant effect of body weight on blood pressure, but not on pulse. When allowance was made for body weight, there were significant effects of both sex and diet on blood pressure, and a significant effect of diet (but not sex) on pulse. There was no interaction between sex and diet for either blood pressure or pulse. Blood pressure in females (118.5 \pm 1.9 mmHg) was significantly greater than in males (110.7 \pm 2.0 mmHg); the relative difference was greatest in the MLPU and MLPA groups and least in the MLP group. There was no difference in blood pressure between the CON and MLPG groups, but blood pressure was

significantly higher in the MLP, MLPU and MLPA groups compared with either CON or MLPG (by approx. 15–20%). There were no differences in blood pressure between the MLP, MLPU and MLPA groups. When the analysis was repeated using the average blood pressure for each litter, rather than individual measurements in each animal, significant differences in blood pressure were found between the MLPG group on the one hand and the MLP ($P = 0.012$), MLPU ($P = 0.012$) and MLPA ($P = 0.03$) groups on the other. The pulse rate did not differ between the CON, MLP, MLPU and MLPG groups, but was significantly slower in the MLPA group compared with other groups, by between 8% and 13%. The pattern of differences in blood pressure and pulse among the different dietary groups was not

similar to the patterns of differences in body weight or in the absolute or relative weights of any of the organs, in animals of either sex or for both sexes combined.

DISCUSSION

The present study shows that a decrease in the protein content of the diet from 18% to 9% during pregnancy resulted in the development of higher blood pressure by 4 weeks of age in the offspring. When non-essential nitrogen was added to the 9% casein diet consumed during pregnancy, there were marked effects on blood pressure, pulse, body weight and organ weight of the offspring, which varied depending upon the form in which the nitrogen was provided. The addition of nitrogen as either urea or alanine had no effect upon systolic blood pressure. However, when the extra nitrogen was provided to the mother in the form of glycine, the blood pressure of the offspring was significantly lower, and similar to the level observed in offspring of mothers given the CON diet during pregnancy. Although there were effects of sex and body size on blood pressure, a highly significant effect of maternal diet remained after allowance had been made for these variables. With regard to pulse rate, the MLPA diet was associated with a lower pulse rate in the offspring compared with any other dietary group. Hence the effects of maternal diet upon blood pressure and pulse in the offspring were not explained by effects of sex, body size or organ size.

Maternal dietary exposure had an important effect upon postnatal growth of the offspring, with the MLP group having the most marked reduction in body weight. MLP rats had relatively large liver and lungs, with proportionate preservation of the brain. Added nitrogen during pregnancy had variable effects. Overall, offspring of the three supplemented groups (MLPU, MLPA and MLPG) had the largest proportionate brain size. Supplementing the diet with urea enabled the MLPU group to achieve a body weight similar to that of the CON group, with proportionate growth of all tissues other than brain. By comparison, the MLPA and MLPG groups weighed less and had disproportionate growth of some tissues. Although liver, heart and kidney development was proportionate in the MLPA offspring, this group had the largest relative brain size, relatively small lungs and the smallest spleen. For the MLPG group there was proportionate growth of heart, kidneys and lung, with a relatively large liver and spleen. These data show that the form in which nitrogen is provided in the diet of the mother during pregnancy can exert a marked effect upon the general growth of the body and specific organs, and on physiological function, of the offspring, but that these changes may be specific for each dietary intervention and in relation to identified function. Of greatest

interest is the fact that the hypertensive effect of a low-protein diet during pregnancy on the blood pressure of the offspring was effectively reversed by supplemental glycine. This effect was not seen when an equivalent amount of nitrogen was provided as either urea or alanine, demonstrating that the beneficial effect on blood pressure is specific. These data suggest that the availability of glycine during pregnancy exerts a specific effect upon the capability of the mother to provide a suitable nutritional environment for the development of the cardiovascular system of her offspring.

In response to a change in dietary protein, amino acid or nitrogen intake, adaptive responses are brought into play that protect essential functions. Central to these responses is a reduction in the excretion of nitrogen to enable nitrogen balance to be achieved, through a decrease in the urinary loss of urea [27]. There may be increased salvage of urea-nitrogen through the metabolic activity of the colonic microflora [16,23], and in rats the practice of refection enables bacterially synthesized amino acids to contribute to the overall dietary quality of the host [24,28]. Thus, although urea is not a particularly efficient source of non-specific nitrogen, it can be used to meet the need at marginal levels of dietary protein consumption [16]. By contrast, the capacity for the endogenous formation of glycine appears marginal on lower-protein diets, and the addition of glycine as a source of NEAA is of particular benefit in correcting any limitation [16,29,30]. Indirect evidence suggests that, in humans, the ability to meet the demands for glycine during pregnancy and infancy is only marginally adequate, and is likely to be stressed on low-protein-containing diets [31]. In the present study, different sources of non-essential nitrogen exerted different effects on the structure and function of the developing fetus. Additional glycine did not confer any benefit in terms of growth in weight or for specific organs, but there was a distinct benefit in terms of blood pressure reduction. By contrast, added urea appeared to offset many of the limitations in terms of general growth of the body and specific tissues, but had no effect at all in reversing the hypertensive effect of the low-protein diet. There was no evidence that supplemental alanine conferred any structural benefit, nor that there was any beneficial effect upon blood pressure.

It will be important to determine the mechanisms through which the relative benefit conferred by glycine operates. Glycine is required for a number of critical metabolic pathways, in which it is consumed as a fundamental building block [32]. Of special importance, among these, are the extraordinary demands for glycine in the synthesis of structural proteins such as collagen and elastin. Glycine is also used specifically to form compounds that carry out critical functional roles during growth and in metabolism, such as nucleotides, haem and glutathione. There is indirect evidence that some or all of

these may be altered during critical stages of growth and development. The placental pathway through which some of the glycine required by the fetus is made available is a folate-dependent enzyme system, and is therefore likely to be sensitive to the folate status of the mother [33]. Folate deficiency is known to limit the production and availability of glycine [34]. Perturbation of folate metabolism through the administration of methotrexate during pregnancy leads to impaired elastin formation in the aorta, with permanent changes in the elastic properties of the great vessels, which would predispose to higher blood pressure in the offspring [35,36]. Similar functional changes have been noted in hypertensive individuals and in the offspring of rats exposed to a low-protein diet during pregnancy [13,37]. There is epidemiological evidence for both the developed and developing world that limitations in dietary folate consumption are associated with small size at birth [38,39].

Rees et al. [15] used the same rodent model with a maternal low-protein diet to explore changes in the availability of individual amino acids to the fetus, with a focus on the extent to which limitation of metabolic interconversions might limit the formation of key metabolic intermediates. They found a similar general pattern of fetal growth and development to that noted previously [40]. On the low-protein diet, the amino acid concentration in maternal serum showed increases for glutamic acid, glutamine and glycine, and a fall for the branched-chain amino acids and threonine, as might be expected in response to such a diet. The only change of note in the fetal pool was a fall in threonine concentration. There are a number of ways in which these concentration differences might be interpreted in terms of dynamic flows of individual amino acids, but one possibility would be that an increase in the concentrations of glutamine and glycine in the maternal circulation would in effect increase the 'drive' for these amino acids to cross a compromised placenta into the fetal compartment. The authors suggest that a limitation in the ability to generate sufficient NEAA might determine the availability to other pathways of a single EAA, such as threonine. Another interaction among EAA and NEAA, which is likely to be of importance, is that between methionine and the glycine-serine-cysteine triplet. In rats the demand for sulphur amino acids to meet the needs for keratin formation for fur are significant, and may not always be met adequately on casein-based diets. In order to prevent any limitation, we added methionine to the diet. In the present study a fixed amount of methionine was provided across each level of dietary protein. Therefore, compared with the CON diet, the MLP diet contains relatively more methionine to nitrogen. Any excess of methionine over the immediate requirement would need to be degraded, and any increased additional flow through the catabolic pathway would increase the utilization of glycine in this process, thereby placing a competitive

demand on the availability of glycine for other pathways [7,41]. In normal non-pregnant women, even the modest addition of dietary methionine to a low-protein diet appears to place a stress on the relative availability of glycine [22]. Therefore it remains possible that methionine or one of its metabolic products (such as homocysteine) is exerting a damaging effect on the maternal, placental or fetal circulation, and that glycine is acting as an effective antidote to this process. In a preliminary report in which the maternal low-protein diet contained more modest amounts of methionine, hypertension was still present in the offspring, suggesting that the level of methionine in the diet might not be the critical factor for the blood pressure effect, but this will need more careful exploration in future studies [42].

The results of the present study indicate that the effect that provision of the MLP diet to the mother has on reducing the weight of the offspring is ameliorated by the addition of urea (MLPU diet), but that there are differences in the responses of individual organs to the changes in the maternal diet. The brain is particularly spared in the MLP group, as are other vital organs, i.e. liver, heart and lung. The response of the spleen, and to a lesser extent the kidney, appears to be more variable among the different dietary groups. The reason for this variability is not immediately clear, but may have implications for the development of immune and renal functions, and will require closer investigation. Overall, it is clear that the response to changes in dietary protein during pregnancy may go beyond considerations that relate to the pattern of EAA consumed, and that different amino acids or sources of nitrogen cannot be seen as being interchangeable. The availability of different forms of nitrogen, and the ability of the mother, placenta and fetus to convert these to a pattern that meets the metabolic demands of the fetus, appear to play an important role in the ability of the fetus to achieve normal growth and development.

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