Maternal Protein Deficiency Causes Hypermethylation of DNA in the Livers of Rat Fetuses[1]

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ABSTRACT Maternal protein deficiency during pregnancy is associated with changes in glucose tolerance and hypertension in the offspring of rats. In this study the growth of rat fetuses was examined when the dams were fed diets containing 18% casein, 9% casein or 8% casein supplemented with threonine. The extra threonine was added to reverse the decrease in circulating threonine concentrations that occurs when pregnant rats are fed protein-deficient diets. The fetuses of the group fed the low protein diet supplemented with threonine were significantly smaller than those of the control group and not significantly different from those fed low protein. Homogenates prepared from the livers of dams fed the diet containing 9% casein oxidized threonine at approximately twice the rate of homogenates prepared from dams fed the diet containing 18% casein. We conclude that circulating levels of threonine fall as a consequence of an increase in the activity of the pathway that metabolizes homocysteine produced by the transulfuration of methionine. Serum homocysteine was unaffected in the dams fed low protein diets compared with controls, but was significantly greater in dams fed the low protein diet supplemented with threonine. Elevated levels of homocysteine are associated with changes in the methylation of DNA. The endogenous methylation of DNA was greater than that of controls in the livers of fetuses from dams fed the 9% protein diets and increased further when the diet was supplemented with threonine. Our results suggest that changes in methionine metabolism increase homocysteine production, which leads to changes in DNA methylation in the fetus. An increase in maternal homocysteine may compromise fetal development, leading to the onset of glucose intolerance and hypertension in adult life. J. Nutr. 130: 1821–1826, 2000.

KEY WORDS: • *rats* • *pregnancy* • *cysteine* • *methionine* • *threonine* • *homocysteine* • *folate*

Epidemiologic studies of human populations have shown that poor growth in utero predisposes an individual to the later development of type-2 (noninsulin-dependent) diabetes mellitus and hypertension in adulthood (Barker and Osmond 1986, Hales and Barker 1992). This phenomenon is not confined to humans; feeding pregnant rats diets moderately deficient in protein has a similar effect, programming the adult blood pressure and glucose metabolism of the offspring (Desai et al. 1995, Langley and Jackson 1994). A diet containing 9% protein is still able to support pregnancy but produces an unusual pattern of fetal growth compared with a control diet containing the optimum of 18% protein. Initially, there is an apparent increase in fetal growth; by d 19, the fetuses of protein-deficient dams are ~7.5% larger than the controls. However, this is not sustained, and by d 21 of the 22.5-d gestation period, the average fetal weight is ~15% less than that of the control (Langley-Evans et al. 1996a, Rees et al. 1999a). The serum concentrations of most essential amino acids are unchanged. L-Threonine is the only essential amino acid that changes significantly; both maternal serum concentrations and fetal body pools fall to less than half of those found in rats fed a control diet (Rees et al. 1999a). These changes in threonine metabolism may be linked to the abnormal fetal growth.

Pregnant rats fed a protein-deficient diet may be unable to maintain normal threonine concentrations either because of a failure in the supply or an increase in demand. If the dam is unable to compensate for the reduction in the dietary intake by mobilizing sufficient threonine from body proteins, then the circulating concentrations will fall. This is easily tested by feeding pregnant rats low protein diets supplemented with additional threonine. An increase in the metabolic demand for threonine will also reduce concentrations in the circulation; the two possible routes of threonine metabolism are shown in **Figure 1**. It can be deaminated by the enzyme threonine-serine dehydratase (EC 4.2.1.16: TDH)[3] to yield 2-ketobutyric acid which is then oxidized to CO_2. Alternatively, L-threonine 3-dehydrogenase (EC 1.1.1.103; TDG) converts it to 2-amino-3-ketobutyrate, which is then cleaved to yield glycine + acetyl-CoA. It can be seen from Figure 1 that both pathways of threonine oxidation are linked to the metabolism of methionine. If growing male rats are fed a diet

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[3] Abbreviations used: SAHcy, *S*-adenosyl homocysteine; SAM, *S*-adenosyl methionine; TDG, L-threonine 3-dehydrogenase; TDH, L-threonine-serine dehydratase.

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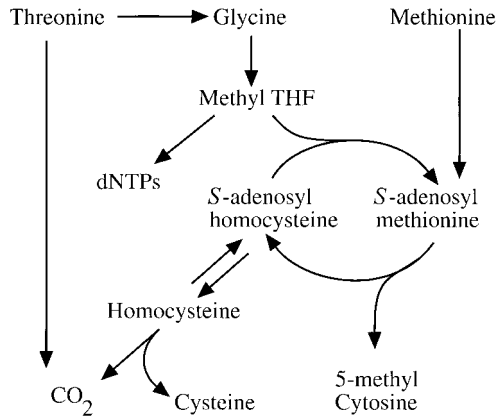

FIGURE 1 Metabolic interactions among threonine, glycine and methionine. Threonine can be metabolized through two different routes. Threonine dehydratase directly deaminates threonine to the ketoacid, which is oxidized to CO_2. Alternatively, threonine dehydrogenase initiates its conversion to glycine, which can be used to form methyl tetrahydrofolate (methyl THF). *S*-Adenosyl homocysteine is formed by transfer of the *S*-methyl group of methionine to a variety of methyl acceptors including DNA (5-methyl cytosine). After hydrolysis to give free homocysteine, the sulfur is transferred to cysteine, and the carbon skeleton is deaminated by threonine dehydratase and oxidized. The fetus is unable to oxidize homocysteine and converts it to *S*-adenosyl methionine (SAM). This remethylation utilizes methyl THF, which is also a precursor for nucleotide and deoxynucleoside triphosphate (dNTP) synthesis.

containing an excess of methionine, there is an increase in the rate of transulfuration; this induces threonine-serine dehydratase, causing plasma threonine levels to fall (Girard-Globa et al. 1972). The change in free threonine levels in pregnant rats fed a low protein diet may therefore be linked to the metabolism of methionine.

Adding additional methionine or homoserine to culture media containing 9.5-d-old rat conceptuses changes the relative amounts of *S*-adenosyl methionine (SAM) and *S*- adenosyl homocysteine (SAHcy) in the fetus and improves growth (Van Aerts et al. 1994 and 1995). A wide variety of cellular methylation reactions including changes in global DNA methylation are dependent on SAM and SAHcy (Wainfan and Poirier 1992). In adult rats, the extent of DNA methylation depends on the composition of the diet (Pogribny et al. 1995). Therefore, maternal homocysteine metabolism may influence the levels of DNA methylation in the fetus. To address the metabolic interactions among threonine, homocysteine and DNA methylation in fetal development, we fed pregnant rats low protein diets supplemented with additional threonine.

MATERIALS AND METHODS

Experimental diets. The experimental diets were identical to those described by Langley-Evans et al. (1996a). The control (18% casein) diet contained the following (g/kg): casein, 180; sucrose, 213; cellulose fiber (solkaflok) 50; cornstarch, 425; vitamin mix AIN-76 (AIN 1977), 5; mineral mix AIN-76, 20; corn oil, 100; choline chloride, 2; and DL-methionine, 5. The low protein diet (9% casein) contained only 90 g/kg casein, with compensating increases in the amounts of sucrose and cornstarch (1:3). The low protein diet supplemented with threonine was based on the same mixture but contained only 8% protein supplemented with 1 g/100 g L-threonine (Ajinomoto, Tokyo Japan). Choline chloride and methionine were from Sigma (Poole, Dorset, UK); the other ingredients were from Special Diet Services (Witham, Essex, UK).

Experimental animals. Female rats of the Rowett Hooded Lister strain from the Institute's breeding colony were fed the experimental diets beginning at 6–7 wk of age. Two weeks later, when they weighed ~230–240 g, the rats were mated with males of the same strain. Mating was confirmed by detection of a vaginal plug and this was denoted d 0. The female rats consumed the same diets throughout pregnancy. In two separate experiments, three groups each of eight rats were fed one of the three diets until d 21 of pregnancy. Some rats were injected via the tail vein with 1.85 MBq L-[U-^{14}C]threonine (8.1 GBq/mmol; ICN Biomedicals Basingstoke, Hants, UK) 1 h before killing. Adult rats were killed by stunning and exsanguination; the fetuses were weighed and killed by decapitation. All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Sample collection and analysis. Organs (liver, kidney, heart and brain) of up to eight fetuses, chosen from each dam at random, were rapidly dissected and weighed. Samples were frozen rapidly in liquid nitrogen and subsequently stored at −70°C. To measure total fetal amino acids, one whole fetus was chosen at random from each dam, homogenized in 2 mL of distilled water, and proteins were precipitated by the addition of 1 mL of 2 mol/L perchloric acid. *Nor*-Leucine (100 μmol/L) was added to all samples to act as an external standard. Details of the amino acid analysis have been given previously (Rees et al. 1999a). Homocysteine was measured by the method of Briddon (1998). Within 5 min of collection, samples of maternal and pooled fetal plasma were treated with 1.2% (v/v) dithiothreitol for 2–5 min, deproteinized with sulfosalicylic acid (final concentration 7%) and analyzed on a Pharmacia Alpha Plus amino acid analyzer (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The column was operated in the lithium form, and amino acids were eluted in a stepwise series of lithium citrate buffers and detected with ninhydrin.

Threonine oxidation by liver homogenates. Maternal livers were removed, weighed and 1- to 2-g samples were homogenized in 2 mL of cold buffer [0.25 mol/L sucrose, 10 mmol/L Tris HCl (pH 7.4) and 1 mmol/L EDTA] per gram of tissue. Debris was removed by centrifugation at 800 × *g* for 5 min at 4°C. Aliquots of the homogenate (200 μL) were mixed with 100 μL of buffer containing unlabeled threonine and 100 μL of buffer containing 1.48 kBq [U-^{14}C] L-threonine (ICN Biomedicals Basingstoke,) or L-[U-^{14}C]-homoserine (Rees et al. 1994). A small tube containing 0.1 mL of 5 mol/L NaOH dispersed over a paper wick was suspended from the rubber stopper used to seal the tube. The reaction was incubated for 2 h at 37°C in a metabolic shaker and terminated by injecting 0.5 mL of 2 mol/L H_2SO_4 through the stopper with a hypodermic syringe. The tubes were returned to the shaker for 2 h. The small tube was then removed, and radioactivity trapped in the NaOH was determined by scintillation counting in 5 mL of Ultima Gold XR scintillant (Packard Bioscience B.V. Groningen, The Netherlands). Homogenates were incubated with a range of threonine concentrations from 0.5 to 20 mmol/L. The rates for each concentration were determined from the linear part of the progress curve, and an apparent V_{max} for each tissue homogenate was determined from reciprocal plots of these data. Protein concentrations were determined by the method of Lowry et al. (1951).

DNA extraction. Tissue (~100 mg) was powdered under liquid nitrogen and suspended in 7 mL of extraction buffer [10 mmol/L Tris-Cl, pH8.0, 0.1 mol/L EDTA, 5 g/L (wt/v) SDS] containing 20 mg/L RNase and 100 mg/L proteinase K (Michalowsky and Jones 1989). The sample was incubated for 12–18 h at 55°C and then extracted three times with water-saturated phenol. DNA was then precipitated from the aqueous phase by the addition of 0.1 volume of 2 mol/L ammonium acetate and 2 volumes absolute ethanol. The sample was then redissolved in TE buffer [10 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA] and dialyzed for 48 h against 100 volumes of TE buffer. DNA was quantified by reading the absorbance at 260 nm.

DNA methyl transferase assay. The extent of CpG methylation was ascertained using the assay of Balaghi and Wagner (1993). Briefly, 0.5 μg of genomic DNA was incubated in a 30-μL volume containing 3 μmol/L (74 kBq) [^{3}H-methyl]*S*-adenosyl methionine (NEN Stevenage, Herts, UK), 3 μL of the 10X reaction buffer and 3 U of SssI DNA methylase (New England Biolabs, Hitchin, Herts, UK). The reaction was incubated at 30°C for 1 h. Aliquots (15 μL) of the reaction mixture were spotted onto DE81 paper circles (Whatman, Maidstone, Kent, UK), washed five times in 0.5 mol/L sodium phosphate buffer (pH 6.8) and dried in air; the remaining radioac-

TABLE 1

Fetal, placental and fetal organ weights on d 21 of pregnancy from rats fed protein-restricted or control diets[1]

	18% casein[2]	9% casein	8% casein + Thr
Dams, *n*	14	14	13
Fetuses/dam	12.8 ± 0.7[a]	14.6 ± 0.4[a]	9.4 ± 0.9[b]
Fetal weight, *g*	4.147 ± 0.128[a]	3.575 ± 0.085[b]	3.766 ± 0.173[b]
Placental wt, *g*	0.572 ± 0.017	0.523 ± 0.014	0.558 ± 0.032
Fetuses, *n*	62	72	53
Liver, *mg*	257.4 ± 5.0[a]	206.5 ± 7.0[b]	208.6 ± 6.3[b]
Kidney, *mg*	38.7 ± 0.7[a]	32.0 ± 0.8[b]	34.0 ± 1.4[b]
Heart, *mg*	18.7 ± 0.4[a]	17.2 ± 0.4[b]	16.5 ± 0.5[b]
Brain, *mg*	166.5 ± 2.7[a]	148.5 ± 2.4[b]	149.0 ± 3.8[b]

[1] Values are means ± SEM. Different superscript letters in a row indicate significant differences. Data were subjected to one-way ANOVA, followed by a Tukey multiple comparison test ($P < 0.05$). All fetuses and placentae from each dam were weighed. Fetal organs were dissected from up to 8 fetuses, chosen at random, from each dam.

[2] Dietary protein sources for the 3 groups were: 18% casein, 9% casein and 8% casein + 1% L-threonine.

tivity was determined by liquid scintillation counting. Blank values were determined from duplicate incubations without enzyme. The reaction had reached its end point within 30 min, and the radioactivity incorporated per microgram of DNA was independent of the amount of DNA present in the reaction mixture.

Statistical analysis. Mean values were calculated for all of the pups and placentae. Previous work with the same strain of rats has not shown any correlation between fetal number and fetal weight (Palmer et al. 1996). Fetal organs were dissected from up to eight fetuses chosen at random from each dam. The data in Tables 1–4 were analyzed by one-way ANOVA followed by a Tukey multiple comparison test. Data were processed using the Instat statistical package (GraphPad Software, San Diego, CA). For the measurements of threonine in Table 2, the variation among fetuses was clearly greater in the supplemented group than in the other two groups. ANOVA was therefore inappropriate for these groups and a two-sample *t* test was used instead. Values are means ± SEM.

RESULTS

Before mating, there were no differences in the food intake and growth rate in the three groups of rats fed the diets containing 18% casein, 9% casein or 8% casein supplemented with threonine. After mating, rats fed the diet containing 18% casein consumed on average 20.8 ± 0.8 g/d for the following 21 d ($n = 8$). The rats fed the diets containing 9% casein and 8% casein supplemented with threonine ate slightly but not significantly more, consuming 23.0 ± 0.8 g/d ($P = 0.06$) and 21.0 ± 0.7 g/d ($P = 0.83$) respectively. On d 21 of pregnancy, the weight of the fetuses from the dams fed the 9% casein diet was 13.7% less than that from the dams fed the 18% casein diet (**Table 1**). In the group fed the diet containing 8% casein supplemented with threonine, fetal weights were 9.1% lower than the controls, significantly smaller ($P = 0.049$) than the group fed the 18% casein diet but not significantly different from those in the group fed the 9% casein diet. This was also the case for individual fetal organs. The weights of livers, kidneys, hearts and brains in the group fed the diet containing 8% casein supplemented with threonine were significantly lower than those of the control group but not significantly different from those of the group fed 9% casein. The placental weights were not affected by protein deficiency or threonine supplementation.

The free threonine concentrations in the fetuses of rats fed the diet containing 9% casein were 31% lower than those in the group fed the diet containing 18% casein (**Table 2**, $P = 0.027$, by Student's *t* test). A similar pattern of differences in free threonine was also observed in the maternal serum (data not shown). Except for increases in glycine and phenylalanine, maternal protein deficiency did not affect the concentrations of other essential amino acids in the fetus. These results are largely consistent with our previous data (Rees et al. 1999a). Free threonine concentrations in the fetuses of dams fed the diet containing 8% casein supplemented with threonine were three times those of the fetuses from dams fed 18% casein (Table 2). In the fetuses of the group fed the diet containing 8% casein supplemented with threonine, the free concentrations of methionine and tryptophan were 53 and 58%, respectively, of the levels found with the 18% casein control diet. There were also smaller differences in most of the other essential amino acids.

In a preliminary experiment, a single rat from each group was injected with L-[U-^{14}C]threonine and killed after 1 h. In all three rats, radioactivity in the maternal serum free amino acid pool and in liver protein was associated almost exclusively with threonine; only 0.02% of the total radioactivity was associated with glycine in the liver proteins and <0.1% was associated with glycine in the free amino acid pool. There was also an unidentified product in the maternal serum, which was not retained by the column of the amino acid analyzer and eluted in the void volume. The characteristics of the unknown product were similar to those of a ketoacid, suggesting that threonine was being oxidized by the maternal liver.

Liver homogenates from nonpregnant rats oxidized L-[U-^{14}C]threonine to $^{14}CO_2$ by a saturable process with an apparent K_m of 13.4 mmol/L (**Fig. 2***A*), which was inhibited by homoserine with an apparent K_i of 3.6 mmol/L (Fig. 2*B*). When radiolabeled homoserine was used as the tracer, the apparent K_m for oxidation was found to be 1.3 mmol/L (Fig. 2*C*), and threonine inhibited with a K_i of 11.2 mmol/L (Fig. 2*D*). The oxidation of threonine and homoserine was also strongly inhibited by cystathionine, another intermediate in the transulfuration pathway with a K_i of 0.1 mmol/L (data not shown). This is consistent with threonine, homoserine and cystathionine sharing a common oxidative pathway through threonine-serine dehydratase. The rate of threonine oxidation by homogenates of the livers of nonpregnant rats fed a diet containing 18% casein was 67.2 ± 7.9 pmol/(min · mg protein) ($n = 4$). By d 21 of pregnancy, this rate had fallen to 18.0 ± 0.1 pmol/(min·mg protein). Homogenates prepared from the livers of dams fed the diet containing 9% casein oxidized threonine at a significantly higher rate, nearly twice that of rats fed the diet containing 18% casein (**Table 3**). The rate of threonine oxidation by liver homogenates from the rats fed the diet containing 8% casein supplemented with threonine did

TABLE 2

Amino acid concentrations from fetuses of dams fed protein-restricted or control diets[1]

	18% casein[2]	9% casein	8% casein + Thr
Fetuses (one/dam), *n*	8	9	7
		nmol/g	
Aspartic acid	18.8 ± 16.3	16.2 ± 9.2	16.5 ± 5.6
Glutamic acid + glutamine	385.0 ± 29.4	415.3 ± 64.0	400.4 ± 97.8
Histidine	41.0 ± 6.7	36.0 ± 3.1	36.0 ± 3.8
Serine	524.1 ± 30.3	620.9 ± 40.4	562.7 ± 27.3
Glycine	1003.1 ± 41.6[b]	1446.6 ± 89.1[a]	1576.2 ± 112.3[a]
Threonine[3]	676.3 ± 75.2[b]	468.3 ± 44.7[c]	2365.2 ± 211.9[a]
Alanine	1767.7 ± 45.6	1759.6 ± 218.1	2895.4 ± 143.7
Arginine	141.7 ± 8.3[a,b]	163.5 ± 6.7[a]	134.2 ± 4.8[b]
Proline	405.4 ± 22.0[a]	422.2 ± 15.3[a]	292.1 ± 52.7[b]
Tyrosine	264.0 ± 13.4[a]	273.6 ± 10.6[a]	198.1 ± 16.1[b]
Valine	282.6 ± 15.3[a]	253.4 ± 8.8[a,b]	211.4 ± 12.6[b]
Isoleucine	106.3 ± 5.7[a]	102.6 ± 5.1[a,b]	80.0 ± 6.3[b]
Leucine	218.0 ± 9.5[a]	208.4 ± 8.2[a,b]	168.4 ± 17.7[b]
Methionine	131.5 ± 17.7[a]	117.6 ± 7.4[a]	63.1 ± 7.5[b]
Tryptophan	59.8 ± 3.3[a]	69.9 ± 3.5[a]	40.4 ± 7.3[b]
Phenylalanine	193.8 ± 7.4[b]	241.6 ± 10.0[a]	190.6 ± 19.3[b]
Lysine	1954.3 ± 108.9[a,b]	2097.1 ± 119.9[a]	1555.0 ± 88.6[b]

[1] Total free amino acids were determined in homogenates of one whole fetus chosen at random from each litter. Values are means ± SEM. With the exception of threonine, data were subjected to one-way ANOVA, followed by a Tukey multiple comparison test ($P < 0.05$). Different superscript letters in a row indicate significant differences.

[2] Dietary protein sources for the 3 groups were: 18% casein, 9% casein and 8% casein + 1% L-threonine.

[3] Data were compared by a 2-sample *t* test.

not differ from that of rats fed the diet containing 18% casein when expressed per milligram protein. However, the threonine-supplemented diet caused a significantly greater liver weight (Table 3). Thus, when expressed per total liver, the total activity was ~40% greater than in rats fed the diet containing 18% casein.

Differences in the activity of threonine serine dehydratase would be expected to affect serum homocysteine concentrations. Levels in the dams fed the diet containing 9% casein were not significantly different from the controls (Table 3). However, maternal serum homocysteine concentrations were significantly greater by 65% in the dams fed the diet containing 8% casein supplemented with threonine. Homocysteine concentrations in fetal serum were lower than those in maternal serum and were not affected by the protein content of the maternal diet or by threonine supplementation.

Treating genomic DNA with SssI methylase and [^{3}H-methyl]-SAM, incorporates [^{3}H]-methyl groups into unmethylated cytosine residues of CpG sites in the DNA. Therefore, there is a reciprocal relationship between the incorporation of label and the extent of endogenous methylation. The incorporation of radioactivity into the livers of fetuses from dams fed the diet containing 9% casein was 22% lower ($P < 0.05$) than in the fetuses of dams fed the diet containing 18% casein (**Table 4**). This indicates that the extent of endogenous DNA methylation in the livers of fetuses from dams fed the low protein diets was greater than in the control. In dams fed the diet containing 8% casein supplemented with threonine, incorporation was further reduced ($P < 0.05$), to 67% of that found in the control, showing that the endogenous DNA methylation was even greater in this group. These differences were confined to the liver; methylation of DNA from the kidneys and hearts of these fetuses was not significantly different from the controls.

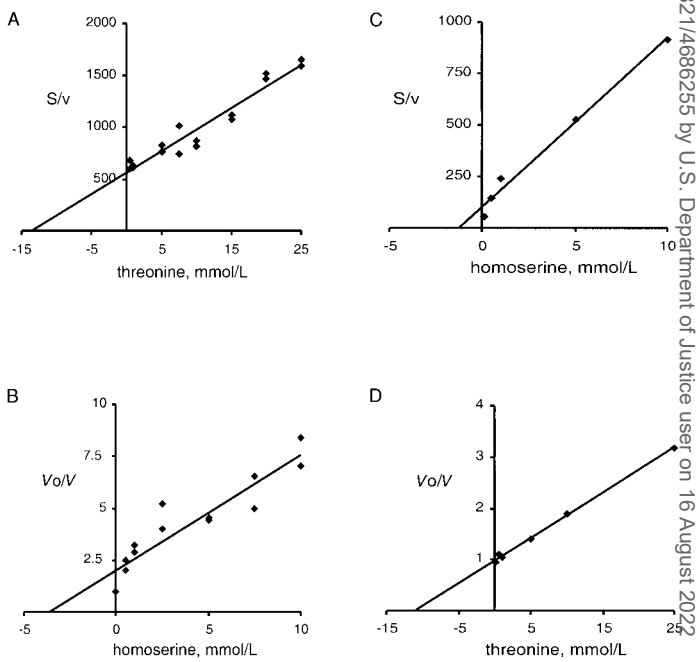

FIGURE 2 The oxidation of threonine and homoserine by homogenates prepared from the livers of nonpregnant female rats fed a standard nonpurified diet. Rates were calculated from the linear portion of the progress curve and the lines fitted by linear regression. K_i was determined from the equation $Vo/V = 1 + ([I]/K_i)$ where Vo is the uninhibited rate for a tracer concentration of substrate and V is the inhibited rate. (*A*) The oxidation of L-threonine, apparent K_m = 13.4 mmol/L; rate = 63.7 pmol/(min·mg protein). (*B*) The inhibition of threonine oxidation by L-homoserine, K_i = 3.6 mmol/L. (*C*) The oxidation of L-homoserine, apparent K_m = 1.3 mmol/L; rate = 203.0 pmol/(min·mg protein). (*D*) The inhibition of homoserine oxidation by L-threonine, K_i = 11.2 mmol/L

TABLE 3

Threonine oxidation by livers of dams fed protein-restricted or control diets[1]

	18% casein[2]	9% casein	8% casein + Thr
Dams, *n*	7	6	6
Liver weight, *g*	10.91 ± 0.59[b]	12.65 ± 0.60[b]	14.14 ± 1.42[a]
Threonine oxidation,[3] *pmol/(min · mg protein)*	18.0 ± 0.1[b]	34.2 ± 0.7[a]	19.7 ± 0.25[b]
Maternal homocysteine,[4] *μmol/L serum*	3.52 ± 0.40[b]	3.44 ± 0.79[b]	5.83 ± 0.86[a]
Fetal homocysteine,[5] *μmol/L serum*	1.83 ± 0.50	0.71 ± 0.31	1.76 ± 0.25

[1] Values are means ± SEM. Different superscript letters in a row indicate significant differences. Data were subjected to one-way ANOVA, followed by a Tukey multiple comparison test ($P < 0.05$).
[2] Dietary protein sources for the 3 groups were: 18% casein, 9% casein and 8% casein +1% L-threonine.
[3] Threonine oxidation was measured in homogenates of maternal livers.
[4] Serum homocysteine was measured in samples from the same dams.
[5] Fetal homocysteine was measured in fetal serum pooled from up to eight pups from the same dam.

DISCUSSION

As with our previous experiments (Rees et al. 1999a), free threonine concentrations in the fetus were lower when pregnant rats were fed a protein-deficient diet. Supplementing the low protein diet with additional threonine failed to restore fetal growth, and the fetuses were similar in weight to those fed the low protein diet alone, suggesting that the threonine supply is not limiting for growth. However, the excess threonine inhibited the uptake of other amino acids; therefore, in the fetuses from dams fed the threonine-supplemented diets, there were effects on most of the essential amino acids. The largest differences were in methionine and tryptophan (53 and 58% of the control, respectively) with much smaller differences in valine and isoleucine (83 and 72% of the control, respectively). Studies of pregnant rats fed diets devoid of single essential amino acids have shown that fetal growth is most sensitive to deficiencies of methionine, valine and isoleucine (Niiyama et al. 1973). Thus, it is possible that any improvement in fetal growth resulting from a restoration of the threonine supply was masked by a deficiency of methionine. However, this is unlikely because the growth of fetuses from dams fed a diet containing 15% lactalbumin, without supplementary methionine, is comparable to the growth of those fed 18% casein despite a methionine concentration of 87.2 ± 3.9 nmol/g (unpublished data).

The casein-based diets used in these experiments are similar to those used in a number of other laboratories to demonstrate the development of hypertension and impaired glucose tolerance in the offspring of female rats fed protein-deficient diets (Desai et al. 1995, Holness and Sugden 1996, Langley-Evans et al. 1996a, Snoeck et al. 1990). Unfortunately, casein provides less than half of the cysteine required by rats during gestation (Reeves et al. 1993). To compensate, extra methionine is added to the diet so that rats can produce cysteine by transulfuration of homocysteine (Fig. 1). Cystathionine and homoserine, by-products of cysteine synthesis, share the same oxidative pathway as threonine; therefore the induction of transulfuration increases threonine oxidation (Girard-Globa et al. 1972). The present results confirm that this same mechanism is operating in pregnant rats; the change in threonine oxidation is an indirect consequence of the change in methionine metabolism. The situation is further complicated because the same methionine supplement (5 g/kg) is added to both the low and high protein diets, supplying the protein-deficient rats with an excess of methionine relative to other amino acids. Supplementing the low protein diet with extra threonine increases circulating homocysteine because it is a competitive inhibitor of homoserine oxidation.

Supplementing protein-deficient diets with taurine reverses the effects of protein deficiency on insulin release by pancreatic islet cells (Cherif et al. 1998). Taurine is derived from cysteine and is particularly important during fetal development. Supplementing with taurine, as well as helping to meet the requirement, will also reduce the demand for cysteine. This in turn will lessen the flux through the transulfuration pathway and reduce total homocysteine production. It is also important to note that the metabolism of methionine is influenced by endocrine factors such as the glucocorticoid hormones (Finkelstein et al. 1978). It has been suggested that maternal glucocorticoids crossing the placenta are an important regulator of fetal development (Benediktsson et al. 1993), and experiments in rats have shown that pharmacologic interventions that alter glucocorticoid status can mimic the effects of protein deficiency (Langley-Evans et al. 1996b). Because these experiments were conducted using casein-based diets, it is possible that changes in sulfur amino acid metabolism may have been involved in the effects observed.

Comparatively small increases in the levels of homocysteine have adverse effects on a number of other physiologic systems, particularly endothelial cell function (Selhub 1999). In humans, elevated levels of homocysteine are associated with obstetric complications including preeclampsia, neural tube defects and recurrent miscarriage (Wouters et al. 1993). The mechanisms are unknown but may be related to disturbed fetal metabolism (Ma-

TABLE 4

DNA methylation of fetal organs from fetuses of dams fed protein-restricted or control diets[1]

	18% casein[2]	9% casein	8% casein + Thr
Fetuses, *n*	13	14	12
	dpm/0.5 μg DNA		
Liver	28545[a] ± 2478	22321[b] ± 1273	19244[c] ± 1208
Heart	9000 ± 429	9414 ± 457	9392 ± 279
Kidney	13744 ± 522	13652 ± 465	12889 ± 941

[1] Values are means ± SEM. Results are expressed as dpm incorporated per 0.5 μg of DNA extracted from the organs of one pup chosen at random from each litter. Different superscript letters in a row indicate significant differences. Data were subjected to one-way ANOVA, followed by a Tukey multiple comparison test ($P < 0.05$).
[2] Dietary protein sources for the 3 groups were: 18% casein, 9% casein and 8% casein + 1% L-threonine.

linow et al. 1998). Because the fetus lacks cystathionine β-synthase, its only means of eliminating homocysteine is through the synthesis of *S*-adenosyl homocysteine, which is then remethylated to SAM (VanAerts et al. 1995). When d-11 rat embryos are cultured ex utero in media containing excess homocysteine, SAM levels are increased, stimulating growth (VanAerts et al. 1994), a result that may explain the early increase in fetal growth seen in pregnant rats fed protein-deficient diets (Langley-Evans et al. 1996a, Rees et al. 1999a). The remethylation of *S*-adenosyl homocysteine also increases the demand for methyl groups derived from tetrahydrofolate, reducing the availability of folates for deoxynucleoside triphosphate synthesis (James et al. 1992). Genes such as p53, which are involved in the response to nucleotide deficiency, are more highly expressed in immature organs (Rees et al. 1999b). The effect of restricting the supply of nucleotides and causing a delay in DNA synthesis is unknown, but this simple growth arrest may be sufficient to program subsequent development.

Cell culture experiments suggest that the fetal programming of adult metabolism is not simply a consequence of altered cell growth. Isolated cells continue to show a permanent change in their function. For example, pancreatic islets from the offspring of protein-deficient animals release less insulin (Cherif et al. 1996), and there are changes in the insulin signaling system of isolated adipocytes (Ozanne et al. 1997). The current experiments show genome-wide changes in DNA methylation, which are apparently confined to the liver. These changes are similar, however, to those seen in adults whose level of DNA methylation can be altered by diet-induced changes in homocysteine and folate status (Jacob et al. 1998).It is possible that these changes may include cytosine residues important for the regulation of specific genes. For example, there are well-characterized changes in the methylation of the insulin-like growth factor II gene, which lead to a loss of imprinting and fetal oversize in mice (Leighton et al. 1995). It remains to be seen whether such highly specific changes in DNA methylation can be caused directly by alterations in the SAM pool.

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