Dietary Protein Restriction of Pregnant Rats Induces and Folic Acid Supplementation Prevents Epigenetic Modification of Hepatic Gene Expression in the Offspring[1]

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ABSTRACT Environmental constraints during early life result in phenotypic changes that can be associated with increased disease risk in later life. This suggests persistent alteration of gene transcription. DNA methylation, which is largely established in utero, provides a causal mechanism by which unbalanced prenatal nutrition results in such altered gene expression. We investigated the effect of unbalanced maternal nutrition on the methylation status and expression of the glucocorticoid receptor (GR) and peroxisomal proliferator-activated receptor (PPAR) genes in rat offspring after weaning. Dams were fed a control protein (C; 180 g/kg protein plus 1 mg/kg folic acid), restricted protein (R; 90 g/kg casein plus 1 mg/kg folic acid), or restricted protein plus 5 mg/kg folic acid (RF) diet throughout pregnancy. Pups were killed 6 d after weaning ($n = 10$ per group). Gene methylation was determined by methylation-sensitive PCR and mRNA expression by semiquantitative RT-PCR. PPARα gene methylation was 20.6% lower ($P < 0.001$) and expression 10.5-fold higher in R compared with C pups. GR gene methylation was 22.8% lower ($P < 0.05$) and expression 200% higher ($P < 0.01$) in R pups than in C pups. The RF diet prevented these changes. PPARγ methylation status and expression did not differ among the groups. Acyl-CoA oxidase expression followed that of PPARα. These results show that unbalanced prenatal nutrition induces persistent, gene-specific epigenetic changes that alter mRNA expression. Epigenetic regulation of gene transcription provides a strong candidate mechanism for fetal programming. J. Nutr. 135: 1382–1386, 2005.

KEY WORDS: • *fetal programming* • *epigenetic regulation* • *transcription factor*

In humans, poor growth during early life has been associated with increased risk of chronic noncommunicable diseases in later life, in particular diseases characteristic of the metabolic syndrome and cardiovascular disease (1). Environmental constraints, e.g., unbalanced nutrition before birth and in infancy, result in metabolic and structural adaptations that lead to persistent modifications to the phenotype of the offspring, i.e., fetal programming (2). The offspring of laboratory animals in which maternal nutrition was restricted during early life exhibit many of the correlates of disease processes associated with reduced growth in early life in humans (3). It was suggested recently that such modification of the phenotype of the offspring may represent adaptive responses that predict the environment in later life and so confer a deferred survival advantage (4). Incorrect prediction results in an inappropriate phenotype and consequently a greater risk of disease.

Although the adverse effects of environmental insults in early life have been documented extensively in epidemiologic studies (1), in whole animals [reviewed in (3)], specific cell types (5), and early embryos (6) the underlying molecular mechanism has not been identified. Persistent alterations to the phenotype of the offspring imply stable changes in gene expression. Candidate genes for such effects arise from studies showing altered gene expression in the offspring of laboratory animals fed restricted diets. For example, maternal protein restriction during pregnancy in rats increased glucocorticoid receptor (GR)[3] expression and reduced expression of 11β-hydroxysteroid dehydrogenase type II (11β-HSD) in liver, lung, kidney, and brain in the offspring (7) and altered peroxisomal proliferator-activated receptor (PPAR) (8) and acetyl-CoA carboxylase and fatty acid synthase expression (9) associated with impaired lipid homeostasis. These genes are of particular interest because perturbations in their expression are associated with disturbances in cardiovascular and metabolic control in animals and humans. GR and PPARs play important roles in embryogenesis (10,11). In adults, GR activity is important for regulation of blood pressure (12), whereas PPAR activities are central to lipid and carbohydrate homeostasis

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[3] Abbreviations used: AOX, Acyl CoA-oxidase; C, maternal control diet; Ct, cycle threshold; GR, glucocorticoid receptor; 11β-HSD, 11β-hydroxysteroid dehydrogenase type II; PPAR, peroxisomal proliferator-activated receptor; R, maternal protein-restricted diet; RF, maternal protein-restricted diet supplemented with folic acid.

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(13,14). Increased glucocorticoid exposure during early life has been implicated in the induction of hypertension (3,7,15), and altered PPAR activity is associated with induction of dyslipidemia (8).

One potential mechanism that could bring about long-term changes in gene expression is altered DNA methylation. Methylation at the 5′ position of cytosine in CpG dinucleotides is a common modification in mammalian genomes and is associated with stable variations in gene expression. Methylation of CpG rich clusters, termed CpG islands, which often span the promoter regions of genes, is associated with transcriptional repression, whereas hypomethylation of CpGs is associated with transcriptional activity (16). Such epigenetic changes to gene expression are critical for normal cell differentiation and embryogenesis (17,18). Because DNA methylation patterns are largely established in utero, the embryonic and fetal environment may alter DNA methylation, inducing stable changes in gene expression that may be sustained throughout the life span of an individual (19). It was shown recently that maternal grooming behavior results in changes in the methylation status and expression of the GR in the hippocampus of rat offspring, producing permanent changes in their stress response (20). Moreover, reducing uterine blood flow in rats alters the methylation status and expression of p53 in the kidney of the offspring (21).

Much of the current interest in the developmental origins of disease in humans concerns the effects of a nutritional mismatch between prenatal and later life [reviewed in (4)]. However, despite strong evidence for candidacy, the effect of maternal undernutrition on the methylation and expression of specific genes in the offspring has not been reported. In the present study, we employed a well-established model (22) to measure the effect of maternal protein restriction during pregnancy on the methylation status and expression of GR, PPARα, and PPARγ in the liver of the offspring after weaning. Moreover, because the rate-limiting enzyme in the peroxisomal β-oxidation pathway acyl-CoA oxidase (AOX) is regulated directly by PPARα (23), we also measured AOX expression in the liver of the offspring. Because the pathway responsible for supply of 1-carbon groups is dependent upon folate metabolism, we also investigated whether the effects of the protein-restricted diet on the methylation status or expression of GR, PPARα and γ, and AOX in liver of the offspring were ameliorated by supplementing the maternal diet with folic acid.

MATERIALS AND METHODS

Rats and diets. All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986). The low-protein diet used was described previously (22). Briefly, virgin female Wistar rats were mated and fed 1 of 3 diets from conception until delivery (each group contained 5 females): control (C; 180 g/kg protein plus 1 mg/kg folic acid); restricted (R; 90 g/kg casein plus 1 mg/kg folic acid) or restricted supplemented with additional folic acid (RF; 90 g/kg casein plus 5 mg/kg folic acid). **Table 1** summarizes the nutrient composition of these diets. After spontaneous delivery at 21 d, litters were reduced to 8 pups and dams that consumed the AIN-76A diet (Special Diets Services) throughout lactation. The pups were weaned onto this diet 28 d after birth and killed by asphyxiation with CO_2 at 34 d. Livers were removed immediately, frozen in liquid nitrogen, and stored at −80°C. From each litter, 2 livers were selected for analysis of gene expression (n = 10 livers/maternal dietary group, 5 males and 5 females).

Methylation-sensitive PCR. The methylation status of genes was determined using methylation-sensitive PCR as described (21). Genomic DNA (5 μg), isolated from the livers of rats, was treated with the methylation-sensitive restriction enzyme Aci1 as instructed by the manufacturer (New England Biolabs). The resulting DNA was then amplified using real-time PCR, which was performed in a total volume of 25 μL with SYBR® Green Jumpstart ready mix as described by the manufacturer (Sigma). Cycle parameters were 55°C × 5 min, 95°C × 10 min, and then 40 cycles of 95°C × 30 s → 60°C × 60 s → 72°C × 60 s. Single bands of the appropriate size were verified by gel electrophoresis. For each gene, PCR primers were designed to amplify the 5′CpG island that spans the promoter region. For GR, the PCR primers used amplified the 307-bp fragment, containing 15 Aci1 sites; this targeted segment of DNA spans the exon 1_{10} promoter, which is the predominant promoter used in liver (24). For PPARγ, the γ 1 promoter region, which is the major promoter used in liver (25), was amplified. A 322-bp segment of the CpG island containing 15 ACi1 sites was amplified. For PPARα, a 369-bp segment of the CpG island that spans the promoter region of this gene immediately 5′ to exon 1, was amplified; this region comprises 17 Aci1 sites. Primer sequences are listed in **Table 2**. CpG methylation at these sites prevents Aci1 digestion and allows the amplification of the promoter fragment, resulting in a low cycle threshold value (Ct value). In contrast, if the CpG island is not methylated, then Aci1 will cleave the DNA and prevent amplification of the fragment, resulting in a high Ct value. Therefore, this technique is sensitive for methylation only within the Aci1 consensus sequence (CCGG). As an internal control, the promoter region from the rat hexokinase I gene, which contains no CpGs and no Aci1 recognition sites, was amplified. All C_T values were normalized to the internal control. Each sample was analyzed in triplicate.

Measurement of mRNA expression by RT-PCR. The levels of PPARα, PPARγ, GR, and AOX mRNA were determined by RT-PCR amplification and quantified by densitometry (8). Briefly, total RNA was isolated from cells using TRIZOL reagent (InVitrogen), and 0.1 μg served as a template to prepare cDNA using 100 U Moloney-Murine Leukemia Virus reverse transcriptase. cDNA was amplified using primers specific to PPARα and γ, GR, and AOX. The PCR conditions in which the input cDNA was linearly proportional to the PCR product were initially established for each primer pair. One tenth of the cDNA sample was amplified for 25 cycles for the housekeeping gene cyclophilin and for 30 cycles for PPARα and γ,

TABLE 1

Composition of diets fed to pregnant and lactating rats

	Pregnancy diets			Lactation diet
	C	R	RF	AIN-76A
	g/kg			
Casein	180	90	90	200
Folic acid	1	1	5	2
Cornstarch	425	482	482	150
Sucrose	213	243	243	500
Choline	2	2	2	2
DL-Methionine	5	5	5	3
Vitamins[1]	5	5	5	5
Minerals[2]	20	20	20	20
Cellulose	50	50	50	50
Corn oil	100	100	100	50
Total metabolizable energy, *MJ/kg*	20.2	19.9	19.9	15.5

[1] Vitamin mix: Thiamine hydrochloride 2.4 mg/kg; riboflavin 2.4 mg/kg; pyridoxine hydrochloride 2.8 mg/kg; nicotinic acid 12.0 mg/kg; D-calcium pantothenate 6.4 mg/kg; biotin 0.01 mg/kg; cyanocobalbumin 0.003 mg/kg; retinyl palmitate 6.4 mg/kg; DL-α-tocopherol acetate 79.9 mg/kg; cholecalciferol 1.0 g/kg; menaquinone 0.02 mg/kg.

[2] Mineral mix: Calcium phosphate dibasic 11.3 g/kg; sodium chloride 1.7 g/kg; potassium citrate monohydrate 5.0 g/kg; potassium sulphate 1.2 g/kg; magnesium sulphate 0.5 g/kg; magnesium carbonate 0.1 g/kg; ferric citrate 0.1 g/kg; zinc carbonate 36.2 mg/kg; cupric carbonate 6.8 mg/kg; potassium iodate 0.2 mg/kg; sodium selenite 0.2 mg/kg; chromium potassium sulphate 12.5 mg/kg.

TABLE 2

Primer sequences used in methylation-sensitive PCR and semiquantitative RT-PCR analysis of genes in the liver of the offspring 34 d after birth

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
	Methylation-sensitive PCR	
GR	CGTCTTGTTCCACCCACT	CCTTGCAGTTGCCGACAG
PPARα	TGTGTCTCGTTCTGAACCG	TCCACCCACCTCACTGTC
PPARγ	CGACTGTGAGGAGCAAGG	CCCAGGTCTCTTCTTCAG
Hexokinase	GAACCTGGACAGGTGTAGGAGAATC	AGCACTAGTGTGTCCCACTGTCC
	Analysis of mRNA expression by semiquantitative RT-PCR	
GR	GGAGAATTATGACCACACTCAAC	GCAGTAGGTAAGGAGATTCTCAA
PPARα	CTGGTCAAGCTCAGGACACA	AAACGGATTGCATTGTGTGA
PPARγ	TGCAGATTACAAGTATGAC	TCGATATCACTGGAGATC
AOX	CCAATCACGCAATAGTTCTGG	CGCTGTATCGTATGGCGAT
Cyclophilin	TTGGGTCGCGTCTGCTTCGA	GCCAGGACCTGTATGCTTCA

GR, and AOX. mRNA expression was normalized using the housekeeping gene cyclophilin and quantified by densitometry using the Quantity 1 program (Bio-Rad Laboratories). All PCR products were verified by restriction mapping and by hybridization with PPARα and γ, GR, AOX, or cyclophilin probes (data not shown).

Statistical analysis. Results are expressed as means ± SD. Comparisons of mRNA expression or DNA methylation between dietary groups were by 1-way ANOVA with Bonferroni's post hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of restricted maternal diet on PPAR and GR gene methylation. Consumption of the R diet during pregnancy resulted in 21% lower CpG methylation in the PPARα promoter in the livers of the offspring compared with the C group ($P < 0.001$) (**Table 3**). In contrast, there was no difference among the offspring of the different maternal dietary groups in the methylation status of the promoter of PPARγ$_1$, the major PPARγ isoform expressed in liver (Table 3). The methylation status of the GR exon 1$_{10}$ promoter was 23% lower in the offspring of the R group compared with the C group ($P < 0.05$) (Table 3).

PPARα promoter methylation was 17% greater in the RF pups than in the offspring of the R group ($P < 0.001$) (Table 3). PPARα methylation did not differ between the C and RF groups. In contrast, feeding the RF diet did not alter the methylation status of PPARγ$_1$ compared with the R and C groups (Table 3). The methylation status of the GR was lower in the offspring of the R group compared with the RF group (26%, $P < 0.01$) (Table 3). The offspring of the C group and the RF group did not differ in GR promoter methylation.

Expression of PPARα and γ, GR, and AOX mRNA. Examples of the results of RT-PCR analysis of mRNA expression are shown in **Figure 1**. Feeding the R diet during pregnancy resulted in greater PPARα mRNA expression in the liver of the offspring compared with the C (945%, $P < 0.0001$)

TABLE 3

Effects of maternal dietary protein restriction and folic acid supplementation during pregnancy in rats on gene promoter methylation and mRNA expression in the offspring 34 d after birth[1]

Gene	Maternal diet			Statistical comparisons[2]			
	C	R	RF	ANOVA (*P*)	C vs. R (*P*)	C vs. RF (*P*)	R vs. RF (*P*)
	DNA methylation						
	% of control						
GR	100.0 ± 5.8	77.7 ± 5.6	102.8 ± 6.5	<0.005	<0.05	—	<0.01
PPARα	100.0 ± 6.7	79.4 ± 4.8	96.5 ± 7.7	<0.0005	<0.001	—	<0.01
PPARγ	100.0 ± 10.1	98.3 ± 11.4	101.0 ± 6.3				
	mRNA concentration						
	% of control						
GR	100.0 ± 44.1	300.8 ± 103.8	155.4 ± 59.3	<0.0001	<0.001	—	<0.01
PPARα	100.0 ± 63.1	1045.0 ± 161.2	166.9 ± 206.4	<0.0001	<0.001	—	<0.001
PPARγ	100.0 ± 6.9	96.9 ± 7.9	96.1 ± 18.8				
AOX	100.0 ± 48.9	317 ± 69.8	106.0 ± 61.5	<0.0001	<0.001	—	<0.001

[1] Values are mean ± SD, $n = 10$ offspring/maternal dietary group.
[2] Probabilities are reported when $P < 0.05$.

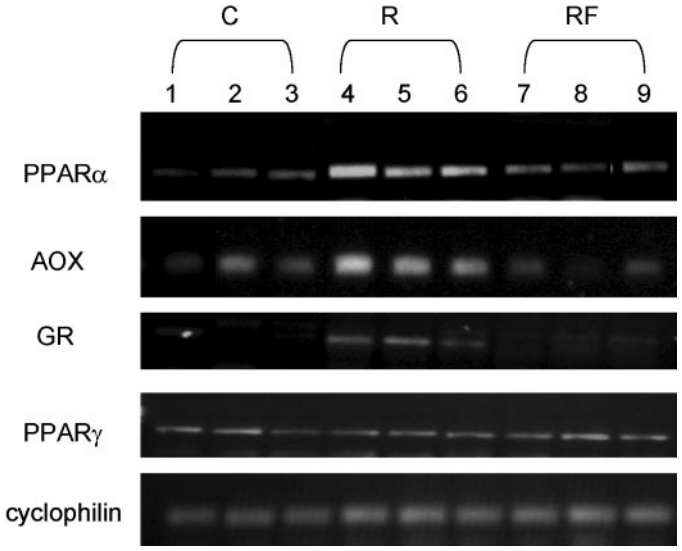

FIGURE 1 mRNA expression by semiquantitative RT-PCR of products from each of 3 livers from the offspring of rats fed control (C), protein-restricted (R), and protein-restricted with folic acid supplementation (RF) diets during pregnancy. Numbers 1 to 9 indicate individual livers. Cyclophilin was used as a housekeeping gene.

and RF groups (526%, $P < 0.0001$) (Table 3). In contrast, PPARα expression did not differ between the offspring of C and RF dams. PPARγ expression did not differ among the offspring of the 3 maternal dietary groups (Table 3). GR receptor expression was greater in the offspring of the R compared with offspring of the C (300%, $P < 0.001$) and RF groups (94%, $P < 0.01$), which did not differ from one another (Table 3).

Because PPARα methylation and expression in the liver of the offspring was influenced by the protein content of the maternal diet, we also investigated whether the expression of AOX, which is regulated directly by PPARα, was altered in this model. AOX expression was greater in the liver of the offspring of the R compared with offspring of the C (217%, $P < 0.001$) and RF dams (199%, $P < 0.001$) (Table 3). However, AOX expression did not differ between the C and RF offspring (Table 3).

DISCUSSION

This study shows for the first time that maternal dietary protein restriction during pregnancy leads specifically to a decrease in the methylation status of PPARα and GR genes in the liver of the offspring after weaning. Because the hypomethylation of the GR and PPARα persisted after weaning, when direct influence of the maternal dietary restriction had ceased, this suggests stable modification to the epigenetic regulation of the expression of these transcription factors. In contrast, methylation of CpG islands in the PPARγ_1 promoter, the major hepatic PPARγ, was not altered by prenatal undernutrition. This suggests that the effects of the maternal diet on the regulation of gene expression in the offspring in rats are gene specific. The observation that maternal undernutrition during pregnancy alters DNA methylation in the offspring is consistent with 2 recent reports. First, experimental reduction of uterine blood flow led to changes in the methylation status of the p53 gene in the kidney of the adult offspring (21). This is important because p53 is involved in the control of apoptosis, which is in turn relevant to renal development, and reduction in nephron number induced in early life has been implicated as a mechanism underlying the early origins of adult hypertension (26). Second, differences in maternal grooming and nursing of the pups in the neonatal period were shown to be associated with permanent changes in the expression of nerve growth factor-1, a transcription factor for GR in the hippocampus of the adult offspring (20). These effects were associated with changes in the adult response to stress, also implicated in cardiovascular and metabolic disease (27). In addition, the maternal behavior-induced effects on the pups could be prevented by administration of the histone deacetylase inhibitor, trichostatin A, to their brains in early life (20). The overall implication of these studies and ours is that changes to the methylation of specific genes in specific tissues underlie the induction of phenotypic changes by environmental cues in early development.

The lower methylation status of the GR and PPARα promoters was associated with substantially greater mRNA expression. This is consistent with previous studies on GR and PPARα expression in this model (7,8). Moreover, because methylation of CpG islands has been firmly established as playing a critical role in transcriptional repression (16), these data suggest a causal relation between hypomethylation of the 5′ CpG islands and increased GR and PPARα expression. However, it should be noted that there is no direct evidence that methylation of the Aci1 sites within the promoters of GR and PPARα suppresses the transcription of these genes. Upregulation of GR activity is associated with DNA demethylation and increased PPARα expression (28). Thus, hypomethylation of the GR promoter resulting in greater GR expression may in part explain the reduced methylation of the PPARα promoter and increased PPARα expression. However, the mechanism by which GR methylation status was reduced is unclear. Furthermore, because PPARα suppresses 11β HSD activity (29), the likely overall effect of impaired regulation of GR and PPARα expression is increased corticosteroid action.

Increased PPARα activity is associated with increased AOX expression (23). In our study, the pattern of AOX mRNA expression mirrored that of PPARα, consistent with reports that AOX is a direct target gene of PPARα. One interpretation is that the increase in the level of PPARα mRNA upregulated AOX expression, thus producing changes to the activity of an effector pathway, peroxisomal β-oxidation. This supports the suggestion that dysregulation of PPARα expression alters the activities of target genes and, potentially, their associated metabolic pathways. However, this does not exclude the possibility that the methylation status of the AOX promoter may also have been modified by the maternal diet. PPARα activity suppresses hepatic Δ6-desaturaese expression (30). Induction of increased PPARα expression is consistent with downregulation of hepatic Δ6-desaturase expression (31) and lower hepatic docosahexaenoic acid concentration (32) in the offspring of the dams fed a protein-restricted diet.

Overall, these changes to the epigenetic regulation of hepatic GR and PPARα expression are consistent with previous reports of induction of later hypertension, and impaired fat and carbohydrate metabolism by maternal protein restriction. Because modifications to the methylation status of gene promoters produce stable alterations in gene expression, this represents a potential mechanism by which insults in early life may lead to persistent changes to the phenotype of the offspring. If so, this mechanism may be directly applicable to understanding the association between patterns of growth in early life and subsequent tissue dysfunction and risk of disease in humans. We should note that the specific effects on indi-

vidual genes in rats would not necessarily be replicated in humans. The results of this study do not indicate whether altered gene methylation occurred prenatally or was due to a persistent effect of undernutrition during pregnancy on the capacity of the dam to satisfy the demands of the offspring during lactation. It remains to be established whether these effects on gene methylation and expression persist throughout the lifespan of the offspring. Because modifications to gene methylation can be passed between generations (33), altered epigenetic regulation of gene expression may also represent one mechanism for intergenerational effects of fetal phenotype induction (34).

Supplementation of the restricted diet with folic acid prevented hypomethylation of GR and PPARα, and the associated increase in the expression of GR, PPARα, and AOX. The observation that maternal folic acid supplementation prevented hypomethylation and the induction of increased GR and PPARα expression suggests that the change in DNA methylation may reflect the impaired supply of folic acid from the mother. There was no effect of supplementation of the restricted diet with folic acid on the methylation status or expression of PPARγ. This suggests the action of homeostatic mechanisms, which allow methylation of hypomethylated genes but prevent hypermethylation of other gene promoters.

Together, these data suggest that 1-carbon metabolism is central to the mechanism of fetal phenotype induction. This also raises the possibility of therapeutic strategies to increase availability of methyl groups and thus prevent or ameliorate the effects of environmental insults in early life. This is supported by the observations that induction of impaired vascular function in rats was prevented by supplementation of the maternal diet with folic acid or glycine (35–37). There is already widespread fortification of foods and the use of folic acid supplements among the population. In view of the fundamental nature and complexity of the effects of folate on the regulation of critical metabolic pathways in the short and longer term, there is an urgent need for more research in this area.

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