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Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPARα promoter of the offspring

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Abstract

Induction of an altered phenotype by prenatal under-nutrition involves changes in the epigenetic regulation of specific genes. We investigated the effect of feeding pregnant rats a protein-restricted (PR) diet with different amounts of folic acid on the methylation of individual CpG dinucleotides in the hepatic PPARα promoter in juvenile offspring, and the effect of the maternal PR diet on CpG methylation in adult offspring. Pregnant rats (n 5 / group) were fed 180g / kg casein (Control) or 90g / kg casein (PR) with 1mg / kg folic acid, or 90g / kg casein and 5 mg / kg folic acid (PRF). Offspring were killed on postnatal d34 (n 5 males and females / group) and d80 (n 5 males / group). Methylation of 16 CpG dinucleotides in the PPARα promoter was measured by pyrosequencing. Mean PPARα promoter methylation in the PR offspring (4.5%) was 26% lower than Controls (6.1%) due to specific reduction at CpG dinucleotides 2 (40%), 3 (43%), 4 (33%) and 16 (48 %) ($P < 0.05$). There was no significant difference in methylation at these CpGs between Control and PRF offspring. Methylation of CpGs 5 and 8 was higher (47% and 63%, respectively, $P < 0.05$) in the PRF offspring than Control or PR offspring. The methylation pattern in d80 PR offspring was comparable to d34 PR offspring. These data show for the first time that prenatal nutrition induces differential changes to the methylation of individual CpG dinucleotides in juvenile rats which persist in adults.

Keywords

Fetal programming; epigenetic; rat; PPARα

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Conflict of interest statement

GCB, KAL and MAH designed the study. ESP, KAL, CT carried out the experimental work. GCB, KAL and MAH wrote the paper with contributions from the other authors. GCB and MAH receive salary support from the British Heart Foundation who also funded part of the study. The remainder of the financial support was from departmental funds. There are no conflicts of interest.

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Introduction

There is an increasing awareness that aspects of the prenatal environment, including nutrition, provide cues which act through developmental plasticity to alter the phenotype of the offspring1. In humans, such developmental cues contribute to the early origins of risk of chronic diseases2. In rats, variations in the phenotype of the offspring are induced by feeding pregnant dams a diet with a moderate reduction in protein3. Induced changes to the phenotype which persist throughout the life-span involve stable alterations to the expression of the genome4. Epigenetic regulation of genes, specifically methylation of clusters of CpG dinucleotides (islands) and covalent modifications of histones in promoter regions, are established during early life and confer stable silencing of transcription which is critical for cell differentiation5. We have shown that feeding pregnant rats a protein-restricted (PR) diet during pregnancy increased glucocorticoid receptor (GR) and PPARα expression in the liver of the offspring by inducing hypomethylation of their respective promoters, possibly by decreasing DNA methyltransferase (Dnmt) -1 expression, and increasing levels of transcription-permissive histone modifications6-8. Hypomethylation and the resulting increase in GR and PPARα expression was prevented by increasing the folic acid content of the PR diet7. In human umbilical cord, Dnmt1, but not Dnmt3a, expression was positively associated with methylation of the GR promoter8. Overall, these findings suggest an epigenetic mechanism by which prenatal nutrition may induce an altered phenotype in the offspring.

The promoter regions of many genes contain several CpG dinucleotides at which induced changes in methylation, and hence epigenetic regulation, might occur. For example, increased pup licking and grooming by lactating rats decreased stress response in the adult offspring by increasing GR expression in the hippocampus through differential hypomethylation of individual CpG dinucleotides in the GR promoter, leading to the altered binding of transcription factors9-11. One example is the binding of NF1A which was regulated by the methylation of a single cytosine9-11. However, it is not known whether maternal under-nutrition during pregnancy alters the methylation of specific CpG dinucleotides within a gene promoter or if all CpGs are affected to the same extent. Furthermore, it is not known whether such patterns of methylation, once induced, are permanent. We have investigated the effect of feeding pregnant rats a PR diet on the methylation of individual CpG dinucleotides in the PPARα promoter in the liver, in both juvenile and adult offspring. We also investigated the effect of increasing the folic acid content of the PR diet on CpG methylation of the PPARα promoter in juvenile offspring.

Materials and methods

Animal procedures

In order to validate assessment of promoter methylation by methylation-sensitive RTPCR which we have used previously7,12 the livers studied here were from the same rats. The sample size at both ages was sufficient to detect significant differences in promoter methylation between dietary groups7,12. All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986). Briefly, Virgin Wistar rats (n 5 per dietary group) were fed from conception to delivery isocaloric diets containing either 180g / kg casein and 1mg / kg folic acid (Control), 90g / kg casein and 1mg / kg folic acid (PR) or 90g / kg casein and 5 mg / kg folic acid (PRF). The composition of the diets has been described previously7. Dams were fed a standard semi-purified diet (AIN 76A; SDS Ltd) from delivery7. Litters were reduced to 8 at birth, equal numbers of males and females, and offspring were weaned onto AIN 76A at d28 and were killed at d34 (juvenile; one liver from each litter was selected for analysis, male to female 3:2) or d80 (adult; males only, one liver from each litter was selected for analysis, n 5 /

group) days. Livers were excised immediately, frozen in liquid nitrogen and stored at −80°C.

Analysis of the methylation status of individual CpG dinucleotides in the hepatic PPARα promoter and PPARα mRNA expression

Genomic DNA was prepared as described7 and bisulphite conversion carried out using the EZ DNA methylation kit (ZymoResearch). The pyrosequencing reaction was carried out by Biotage. Modified DNA was amplified using hot start Taq DNA polymerase (Qiagen) with the following primers; forward GGGGTGTGTTTAGTTTTGAAT, reverse TCACCCCTATCCTAAAACC. PCR products were immobilised on streptavidin-sepharose beads (Amersham) washed, denatured and released into annealing buffer containing the sequencing primer GGGATTTAGTAGGGGA (Biotage). Pyroseqeuncing was carried out using the SQA kit on a PSQ 96MA machine (Biotage) and the percent methylation calculated using the beta version of the Pyro Q CpG software from Biotage. Assay precision was between SD 0.8% to 1.8% and detection limits 2% to 5% methylation. The location of the CpG island in the PPARα promoter is shown in Figure 1A and the sequence in Figure 1B. Putative transcription factor binding sites were deduced using Genomatix Matinspector (Genomatix Software GmbH).

In order to assess the extent to which the methylation status of individual CpGs is associated with PPARα mRNA expression we carried out correlation analysis using data published previously where details of the analytical methods are described7,12.

Statistical analysis

Values are mean (SD) methylation for individual CpG dinucleotides expressed relative to the Control offspring. For the three groups of offspring studied at 34 days, values within each CpG were compared by 1-way ANOVA with Dunnett's *post hoc* analysis. For the two groups of male offspring studied at 80 days, statistical comparisons were by Student's unpaired t-test. Calculation of Pearson's correlation coefficient was used to determine the relationship between methylation status of individual CpGs in the PPARα promoter and mRNA expression. Results were combined irrespective of age or maternal diet in order to provide sufficient data points for correlation analysis.

Results

The results of analysis of the methylation status of the PPARα promoter at d34 are summarised in Figure 1C. 1-way ANOVA showed that there were significant differences in methylation between maternal dietary groups at CpGs 2, 3, 4, 5, 8, and 16 ($P < 0.05$) and a non-significant trend ($P < 0.1$) at CpGs 7, 10 and 11. Mean methylation of the PPARα promoter in offspring of the dams fed a PR diet during pregnancy (4.5%) was significantly lower (26%, $P<0.05$) than Controls (6.1%) due to selective reduction in methylation ($P < 0.05$) at CpGs 2 (40 %), 3 (43 %), 4 (33 %) and 16 (48 %). There was no significant difference in the methylation of these CpG dinucleotides between Control and PRF offspring. There was a non-significant trend ($P < 0.1$) towards lower methylation at CpGs 7, 10 and 11 in the offspring of the PR vs. Control dams, but not in the offspring of dams fed the PRF diet. Although there was no significant difference in methylation at CpGs 5 and 8 between offspring of Control and PR dams, methylation was significantly higher ($P < 0.05$) in the offspring of the dams fed the PRF diet compared to Controls (47 % and 63 %, respectively).

There was no significant difference in the mean level or CpG-specific methylation in the PPARα promoter between offspring of Control dams at d34 and d80 (Figure 1 C and D).

Mean promoter methylation was 28% (P<0.05) lower in the d80 PR offspring (5.1%) compared to Controls (7.1%). The methylation status of specific CpG dinucleotides in the hepatic PPARα promoter was significantly lower in the offspring of PR dams (CpG 2, 72 %; 3, 16 %; 4, 23 %; 7, 42 %; 9, 39 % and 16, 51 %; $P < 0.05$) compared to the offspring of the Control dams (Figure 1D). Mean difference between offspring of Control and PR dams in methylation across all CpGs measured was 39%.

We have published previously the level of PPARα mRNA expression in these samples7. There was a significant negative relationship between the methylation status of CpG 3 and 16 (both P<0.05) such that variation in the level of methylation at CpG 3 predicted 43% and at CpG 16 predicted 39% of the difference between individuals in PPARα mRNA expression.

Discussion

The results of this study show for the first time that feeding a PR diet to pregnant rats induces hypomethylation of specific CpG dinucleotides in the hepatic PPARα promoter in juvenile offspring. This pattern of cytosine hypomethylation was also found in adult male offspring. In d34 offspring, the PRF diet prevented hypomethylation of these CpG dinucleotides, but induced hypermethylation of two other CpGs.

The present findings agree with our previous data which showed using methylation-specific RTPCR that feeding a PR diet to pregnant rats induced an overall 26% reduction in methylation of the PPARα promoter in the liver of the juvenile and adult offspring7,12. The level of methylation within the PPARα promoter region was low (4% to 10%) compared to that reported by Weaver *et al*.11 for GR in the hippocampus (14% to 58%). However, we did find significant differences in the level of methylation of individual CpGs between the Control and PR offspring. Moreover, absolute levels of methylation of specific CpG dinucleotides in the PPARα promoter had a significant negative relationship with mRNA expression. This suggests the magnitude of variation in CpG methylation was sufficient to alter transcription7 and is consistent with the suggestion that variations in relatively low levels of methylation, compared to imprinted genes, allow fine control of transcription by changing the balance in transcription factor regulation13. Such subtle changes in epigenetic regulation are more consistent with the graded changes in phenotype induced by the early life environment than the gross phenotypic changes caused by large changes in the methylation of imprinted genes.

The CpG dinucleotides in the PPARα promoter coincided with the putative binding sites of a number of transcription factors which have an important regulatory role in a wide range of cellular processes (Figure 1B). It is possible that that the differences in methylation of specific CpG dinucleotides observed may change the regulation of transcription in response to individual transcription factors and consequently the capacity of the tissue to respond to a metabolic challenge. Interestingly, the two CpGs which showed an association between methylation and mRNA expression are located within putative binding sites for the transcription factors specificity protein 1 (SP1) and winged helix protein (WHNF), respectively. However, the transcription factor binding sites in Figure 1B are illustrative and the precise effect of differences in CpG methylation between offspring from different maternal dietary groups on transcription factor binding awaits experimental investigation.

The present findings agree in both direction and overall magnitude of effect with our previous data which showed using methylation-specific RTPCR that feeding a PR diet to pregnant rats induced overall hypomethylation of the PPARα promoter in the liver of the juvenile and adult offspring7,12. These data also show that prenatal under-nutrition induced

hypomethylation of specific CpG dinucleotides, rather than altering the methylation of all CpGs in the PPARα promoter. This is in agreement with the effects of maternal nursing behaviour on the epigenetic regulation of GR11. Together these studies suggest that very different aspects of the early life environment induce highly specific changes to the epigenotype of the offspring.

Feeding the PRF diet to pregnant rats prevented hypomethylation of CpGs which showed reduced methylation in the PR offspring, which is consistent with our previous findings7. However, CpGs 5 and 8 were hypermethylated in the PRF offspring which suggests that there may be subtle effects of increased maternal folic acid intake which were not detected by overall assessment of promoter methylation, but which may still alter gene function.

Dnmt1 expression and binding of specific transcription factors is reduced in the liver of the offspring of dams fed a PR diet during pregnancy, while this is prevented by increasing the folic acid content of the PR diet8. Since Dnmt1 is targeted to specific genes14 and CpG dinucleotides15, altered Dnmt1 expression may provide a mechanism for induction of hypomethylation of specific genes and individual CpGs, although how such targeting may occur is not known.

One key principle of the developmental origins of disease hypothesis is that phenotypic characteristics induced in early life persist into adulthood2. We have shown that overall changes in the methylation of the GR and PPARα promoters persist in adult offspring12. The findings of the present study show that the pattern of methylation of specific CpG dinucleotides in the hepatic PPARα promoter in adult offspring of PR dams compared to Controls were essentially the same as in juvenile offspring. This suggests that the pattern of methylation of individual CpGs induced in early life persists into adulthood.

Together these results show that nutrient constraint before birth induces persistent CpG-specific changes to the epigenetic regulation of the PPARα promoter and that these changes are associated with altered mRNA expression. If this were to occur in humans, the precise pattern of CpG methylation may provide one mechanism for graded differences in phenotype induced by the developmental environment which result in differential risk of disease.

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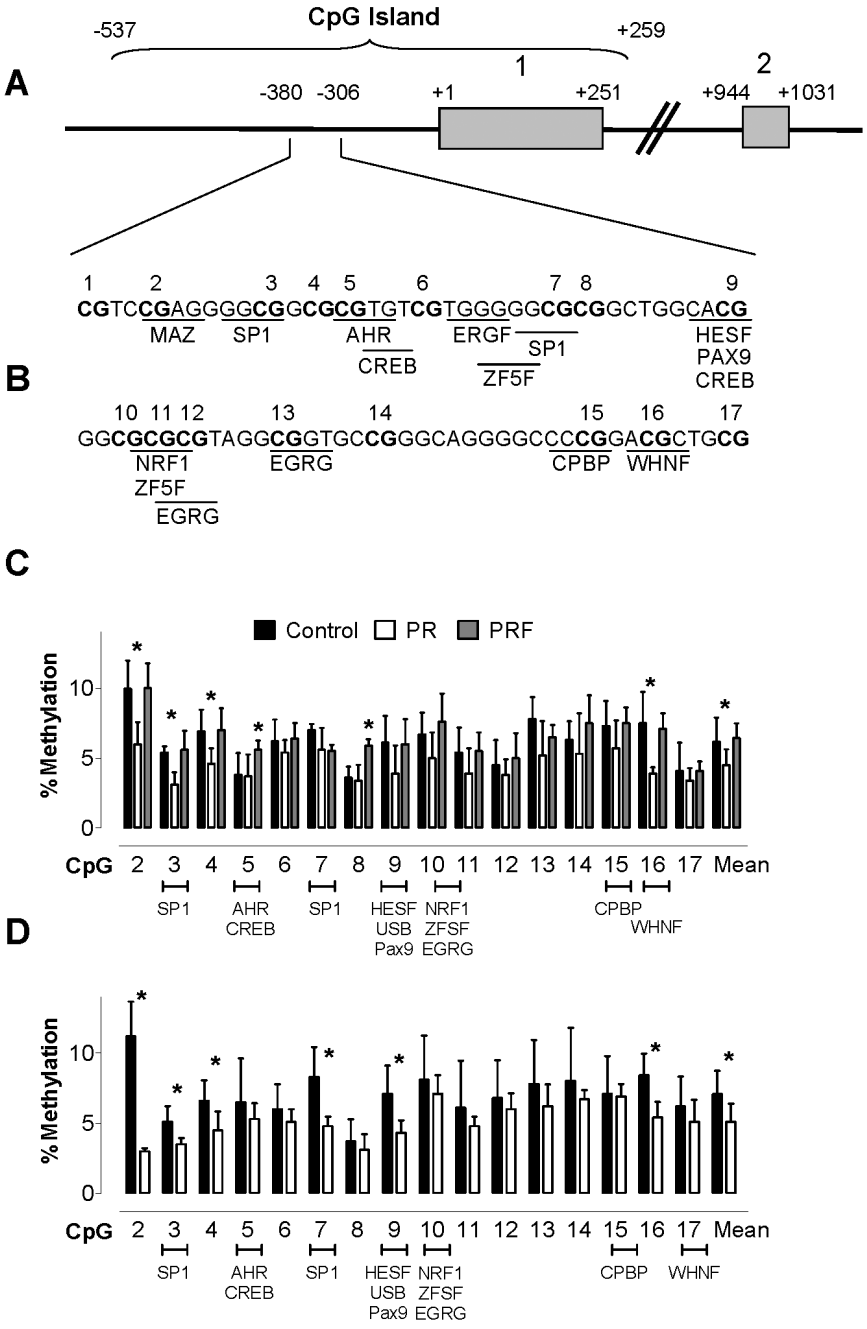

Figure 1.

(A) Structure of the PPARα gene (www.ensembl.org, gene identity number ENSRNOG00000021463). The location of the CpG island was identified using Methprimer (www.urogene.org/methprimer). (B) Nucleotide sequence of the CpG island showing individual CpG dinucleotides and putative transcription factor binding sites. (C) and (D). Methylation of individual CpG dinucleotides in the PPARα promoter in the liver of the offspring of rats fed either a Control, protein restricted (PR) or PR with increased folic acid content (PRF) diet during pregnancy. Data are expressed as mean (SD). (C) d34 male and female offspring (n 5 / group), *indicates values significantly different ($P < 0.05$) from the Control group by 1-way ANOVA with Dunnett's *post hoc* test. (D) d80 male offspring (n 5 /

group), *indicates values significantly different ($P < 0.05$) from Control by Student's unpaired t-test. AHR, aryl hydrocarbon receptor; CPBP, Core promoter-binding protein; CREB, cAMP responsive element binding protein; EGRF, Wilms tumour factor; HESF, hey like transcriptional repressor; NAZ, Myc associated zinc finger protein; NRF1, Nuclear respiratory factor 1; SP1, specificity protein 1; WHNF winged helix protein; ZF5F zinc finger domain transcription factor.

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