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The liver X-receptor gene promoter is hypermethylated in a mouse model of prenatal protein restriction

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van Straten EME, Bloks VW, Huijkman NCA, Baller JFW, van Meer H, Lütjohann D, Kuipers F, Plösch T. The liver X-receptor gene promoter is hypermethylated in a mouse model of prenatal protein restriction. *Am J Physiol Regul Integr Comp Physiol* 298: R275–R282, 2010. First published November 4, 2009; doi:10.1152/ajpregu.00413.2009.—Prenatal nutrition as influenced by the nutritional status of the mother has been identified as a determinant of adult disease. Feeding low-protein diets during pregnancy in rodents is a well-established model to induce programming events in offspring. We hypothesized that protein restriction would influence fetal lipid metabolism by inducing epigenetic adaptations. Pregnant C57BL/6J mice were exposed to a protein-restriction protocol (9% vs. 18% casein). Shortly before birth, dams and fetuses were killed. To identify putative epigenetic changes, CG-dinucleotide-rich region in the promoter of a gene (CpG island) methylation microarrays were performed on DNA isolated from fetal livers. Two hundred four gene promoter regions were differentially methylated upon protein restriction. The liver X-receptor (Lxr) alpha promoter was hypermethylated in protein-restricted pups. Lxr alpha is a nuclear receptor critically involved in control of cholesterol and fatty acid metabolism. The mRNA level of *Lxra* was reduced by 32% in fetal liver upon maternal protein restriction, whereas expression of the Lxr target genes *Abcg5*/*Abcg8* was reduced by 56% and 51%, respectively, measured by real-time quantitative PCR. The same effect, although less pronounced, was observed in the fetal intestine. In vitro methylation of a mouse Lxra-promoter/luciferase expression cassette resulted in a 24-fold transcriptional repression. Our study demonstrates that, in mice, protein restriction during pregnancy interferes with DNA methylation in fetal liver. *Lxra* is a target of differential methylation, and *Lxra* transcription is dependent on DNA methylation. It is tempting to speculate that perinatal nutrition may influence adult lipid metabolism by DNA methylation, which may contribute to the epidemiological relation between perinatal/neonatal nutrition and adult disease.

programming; epigenetics; CpG island methylation microarray

AN OVERWHELMING BODY OF EVIDENCE links fetal (mal)nutrition to the development of chronic diseases at adult age [developmental origins of health and disease hypothesis (4, 5)]. Epidemiological data show that children small for gestational age, who were undernourished during intrauterine development, have a higher risk of developing cardiovascular diseases or the metabolic syndrome in adulthood (3). In humans, fetal malnutrition is related to external factors (starvation, malnutrition, drug consumption of the mother) or to internal factors, such as placental dysfunction leading to reduced routing of nutrients to the fetus (32).

Knowledge of underlying mechanisms of metabolic programming may help to design strategies to halt the current epidemic in metabolic diseases. For this purpose, several animal models have been developed (34). Maternal dietary protein restriction is a well-characterized protocol to mimic fetal malnutrition in laboratory animals, from rodents to sheep (34). Moderate protein restriction (i.e., from 18–20% to 8–10%) in an isocaloric diet has been shown to increase the susceptibility to develop hypertension, insulin resistance, obesity, or dyslipidemia in rodents, especially when "second hit" strategies, such as feeding a high-fat or high-salt diet at adult age are superexposed.

Several explanations for the various observed facets of the long-term consequences of fetal malnutrition have been proposed. For some aspects, morphological changes during early development may play a role, such as reduced nephron numbers as a predeterminant of adult hypertension (13). In recent papers, epigenetic mechanisms like DNA methylation have been proposed to be involved in metabolic programming. In a series of elegant experiments, Lillycrop and colleagues (7, 12, 22–24) have demonstrated that dietary protein restriction can modify the promoter methylation pattern of selected genes involved in metabolic processes and ultimately change gene expression. These authors proposed changes in cellular methyl group metabolism as the underlying mechanism for differential DNA methylation, as the process could be prevented by addition of folate to the protein-restricted diet (22). A genome-wide approach has not been published so far.

CG-dinucleotide-rich regions in the promoter of a gene (CpG islands), have been shown to be of crucial importance for the transcriptional activity of particular promoters (28). In general, transcription of a gene is blocked when the CpG island is methylated. This phenomenon has been extensively studied in tumor biology as methylation of tumor supressor gene promoters or demethylation of protooncogen-promoters may be involved in oncogenesis (25). A useful approach to assess epigenetic changes in tissues involves the application of differential methylation hybridization (DMH) (14). For this, DNA isolated from tissues of interest and adequate controls is treated with methylation-sensitive restriction enzymes. Hypermethylated DNA is protected from enzymatic digestion and is therefore overrepresented in the DNA sample. This difference in abundance can be detected by microarray technology in a genome-wide manner (36).

A detailed characterization of the effects of maternal dietary protein restriction on the developing mouse fetus is a prereq-

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uisite for our understanding of metabolic programming. In the present study, we applied DMH technology to identify genes involved in metabolic programming by fetal malnutrition. C57BL/6J mice were fed control (18% protein) or a protein-restricted diet (9%) throughout pregnancy. Shortly before birth, the animals were killed, and fetal liver DNA was analyzed using a 4.6K mouse CpG island microarray (36). Overall, 137 genomic regions were identified to be hypermethylated and 145 to be hypomethylated upon dietary protein restriction. We characterized one of the metabolically most interesting hypermethylated genes, i.e., the liver-X-receptor (Lxr)-α, in detail. Lxr alpha (or Nr1h3) is a nuclear receptor crucially involved in cellular lipid homeostasis (35). Lxr alpha is activated by oxysterols, binds to its heterodimeric partner the retinoid X-receptor (RXR), and then activates a broad variety of target genes (15). Activation of the LXR target gene *Srebp1c* results in increased lipogenesis required for formation of cholesteryl ester (17). Finally, expression of the adenosine triphosphate-binding cassette (ABC) transporters *Abca1*, *Abcg5*, and *Abcg8* is regulated by LXR, leading to increased removal of sterols out of the cell (27). Therefore, Lxr alpha is a key regulator of lipid homeostasis.

Our DMH/DNA microarray experiment identified the *Lxra* promoter as a target of DNA hypermethylation in a protein-restriction model. Consequently, the purpose of this study was to characterize the effects of *Lxra* promoter hypermethylation on the expression of *Lxra* and its target genes in the mouse fetus and to link it to fetal lipid metabolism. Moreover, we performed extensive in vitro studies both with endogenous *Lxr* and *Lxr*-promoter/luciferase constructs to pinpoint the relation between DNA methylation and gene expression on the molecular level.

MATERIALS AND METHODS

Animals. C57BL/6J mice were obtained from Harlan (Horst, The Netherlands). Animals were housed in temperature-controlled rooms (23°C) with 12:12-h light-dark cycling and free access to standard pellet diet and water ad libitum. To exclude sex-specific effects, only male fetuses were used for all of the analyses described. Sex of the fetuses was determined by PCR as previously described (20, 33). Experimental procedures were approved by the local ethics committee for animal experiments of the University of Groningen.

Experimental procedures. All females received the control diet (180 g casein/kg; Arie Blok, Woerden, The Netherlands) 2 wk prior to mating. Virgin females were time mated. After confirmation of mating by the appearance of a vaginal plug, the females were allocated to be fed either the control diet or a low-protein diet (90 g casein/kg). The diets are described in detail elsewhere (21). At *day 19.5* of gestation, females were anaesthetized with isoflurane and killed by cardiac puncture. Blood was collected in EDTA-containing tubes. Organs were snap frozen in liquid nitrogen and stored at −80°C. Fetuses were removed from uteri, weight and length were measured, and fetuses were killed and dissected. The fetal tissues were randomly assigned to the different follow-up procedures to avoid bias by fetal size or position in the uterus. One male fetus per dam was used for DNA and RNA isolation, one for RNA only, and the remaining ones were used for sterol analysis. Blood samples were taken by exsanguination. Organs of fetuses were collected, immediately snap frozen in liquid nitrogen, and stored at −80°C.

CpG island microarray. DNA was isolated from fetal liver samples by using the QuiAmp DNA minikit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The CpG island microarray was performed following the procedures described by Yan et al. (36). Briefly, fetal DNA (1–2 μg) from five fetuses per experimental group was restricted with *Mse*I (New England Biolabs, Frankfurt am Main, Germany) to fragments of 200 base pairs on average. The number of samples ($n = 5$) was calculated according to Allison et al. (2). The DNA fragments were ligated to universal linker primers (H-12, 5′-TAATCCCTCGGA-3′ and H-24, 5′-AGGCAACTGTGCTATC-CGAGGGAT-3′) and cut with the methylation-sensitive restriction enzymes *Bst*UI and *Hpa*II (New England Biolabs). Fragments containing methylated sites are protected against restriction and will hence be relatively enriched by this procedure. The products were then PCR-amplified using the H-24 primer. As demonstrated by Yan et al. (36), this will result in proportional amplification of the undigested fragments. After purification, the PCR product was coupled to Aminoallyl-dUTP and labeled with Alexa Fluor 647 dye (protein-restriction diet group) or Alexa Fluor 555 dye (control diet group), respectively. The samples were purified and hybridized overnight to a mouse CpG island microarray containing 4,642 clones (obtained from the Sanger Institute) (36). After scanning (Axon 4000A), slide normalization was performed using a subgrid intensity-based method (GeneTraffic; Iobion). The generated tiff images were used to quantify the spot intensities. The latter steps were done at the Ontario University Health Network Microarray Centre (Toronto, Canada). Data were analyzed by the SAM software package (31) as described below. To restrict the results to CpG islands in the proximity of gene promoters, we limited the results to clones within 4,000 base pairs distance to a transcription start site. Finally, the results were annotated and sorted by the gene ontology term Biological Process level 4 (BP4), using DAVID software (10). Information regarding the clones represented on the CpG island microarray can be obtained from the Ontario University Health Network Microarray Centre: http://data.microarrays.ca/cpgmouse/. The CpG island microarray data discussed in this publication have been deposited in the Gene Expression Omnibus, accession no. GSE15280.

Gene expression analysis. TriReagent (Sigma, St. Louis, MO) was used to isolate total RNA from fetal livers and intestines according to the manufacturer's instructions. RNA was quantified using a spectrophotometer (model ND1000; NanoDrop Technologies, Wilmington, DE). cDNA synthesis was performed as described (26). Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector according to the manufacturer's instructions. Primers and probes were obtained from Invitrogen (Breda, The Netherlands) and Eurogentec (Seraing, Belgium), respectively. Primers and probe sequences can be obtained at http://medgen.ugent.be/rtprimerdb/, with the exception of DNA methyltransferase 1 *Dnmt1* (accession no. NM_010066.3: sense, 5′-TGGTGCTGAAGCTCACACTG; antisense, 5′-CCATACTGTCCAGCCTGGAG-3′; probe, 5′-CTGCCTG-GTCCGCATGGGCTAC-3′), *Dnmt3a* (accession no. NM_007872.4, isoform 1 and 2: sense, 5′-CGGCAGAATAGCCAAGTTCA-3′; antisense, 5′-CTGGTCTTTGCCCTGCTTTA-3′; probe, 5′-CAAAGT-GAGGACCATTACCACCAGGTCAAAC-3′); and *Dnmt3b* (accession no. NM_00100361, isoforms 1 to 4: sense, 5′-CCAGTCTTGGAGG-CAATCTG-3′; antisense, 5′-CTGGAGACCTCCCTCTTAGACAG-3′; probe, 5′-CACCAGAGACCAGAGGCCGCAG-3′). Expression levels were normalized to those of 18S ribosomal RNA, which was analyzed in separate runs.

Inhibition of DNA methylation by zebularine. The mouse hepatoma cell line Hepa1.6 was grown in DMEM (Gibco, Breda, the Netherlands), 10% FBS (Gibco), under standard cell culture conditions. One day before treatment, 1 million cells/10-mm dish were seeded. Cells were incubated for 72 h with (in μM): 0 , 20, 50, 100, or 200 of the DNA methylation inhibitor 1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-1 (zebularine; Sigma). Genomic DNA and RNA were isolated with TriReagent (Sigma) as described by the manufacturer.

Bisulfite sequencing of the Lxra promoter. To characterize the methylation pattern of the *Lxra* promoter, bisulfite sequencing was performed (11). DNA was treated with bisulfite by using the EZ DNA methylation kit (Zymo Research, Orange, CA), according to the

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manufacturer's instructions. The *Lxra* promoter was amplified using the primers 5′-AGGGAGGTTGGGAATATAGGTT-3′ and 5′-CTA-CCAAAAAATCCTTCCTACTAA-3′. PCR products were cloned into the pCR2.1 topo vector (Invitrogen), and several randomly picked clones from independent biological samples were sequenced. Sequences obtained were analyzed with the QUMA software package (19) by using the standard setting (upper limit of unconverted CpHs: 5; lower limit of %converted CpHs: 95; upper limit of alignment mismatches: 10; lower limit of %identity: 90). The difference of overall methylation between groups was calculated by the Mann-Whitney *U*-test.

Functional LXR promoter analysis. A region of the *LXRa* promoter from −926 to +413 was amplified by PCR using the primers 5′-GGCAAAACTATCATCATCGTCGTC-3′ and 5′-TTGCTGGG-AGAAGAGGGTGTTGC-3′ and cloned into the promoterless CG-free luciferase vector (pCpGl-basic) (18). This construct was methylated by *SssI* methylase (New England Biolabs). The CG-free cytomegalovirus (CMV)-promoter-luciferase vector pCMV-CpGl was used as control as described before (18). The CpG vectors were kindly provided by Dr. Maja Klug and Dr. Michael Rehli, University Hospital Regensburg, Germany.

COS7 (African Green Monkey kidney fibroblast cell line) and HepG2 (human hepatocellular carcinoma cell line) cells were grown at 0.1% gelatine (Sigma) in DMEM supplemented with 10% FBS (Gibco) under standard cell culture conditions. All constructs were transfected using Fugene (Roche, Mannheim, Germany) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega, Leiden, the Netherlands) as described by the manufacturer, and the luciferase activity was determined using 1% Triton X-100 (Sigma), 25 mM glycyl-glycin (Sigma), 15 mM $MgSO_4$ (Merck, Amsterdam, the Netherlands), 5 mM ATP (Roche), and 6.25 μM D-Luciferin (Sigma) in a Berthold Microplate Luminometer (Berthold, Pforzheim, Germany). Luciferase was normalized to B-galactosidase activity to correct for transfection efficacy.

Sterol analysis. Liver specimens were dried, and the weight was noted before extracting sterols and oxysterols with chloroform/methanol. Briefly, sterols and oxysterols were extracted by cyclohexane after alkaline hydrolysis of the corresponding esters. After evaporation, the hydroxyl groups of the sterols were silylated and transferred into the gas-chromatography vials. Cholesterol was determined by gas chromatography, cholesterol precursors, plant sterols, and oxysterols by gas chromatography-mass spectrometry as previously described (30).

Statistics. Statistical analyses were performed using SPSS version 14.0 for Windows (SPSS, Chicago, IL). Differences between the groups were analyzed by the Mann-Whitney *U*-test. A P value < 0.05 was considered statistically significant. Data presented are as means ± SD.

For the analysis of the CpG island microarray data, a modified *t*-test using Statistical Analysis of Microarrays (SAM) was used. SAM is a variant of permutation analysis developed for microarray analysis and extensively described by Tusher et al. (31). The input to SAM is the normalized signal ratio for each clone from a set of microarrays and a response variable (diet) from each experiment. SAM tests whether the mean clone signal differs from zero. SAM also computes the false-discovery rate (FDR) value in this experiment. FDR was set to 1% by adjusting the delta value (cut off). SAM computes a statistic D value (D-score) for each clone, measuring the strength of the relationship between clones and the diet variable. A list of significant altered clones (FDR <1%) was generated.

RESULTS

Maternal protein restriction has limited effects on fetal biometric parameters. We applied a mild, well-characterized protein-restriction protocol (9% vs. 18% casein) to pregnant C57BL/6J mice. Protein restriction did not lead to differences in body weight, liver size, or plasma total cholesterol concentrations in the dams (Table 1). Litter sizes were identical in both treatment groups (Table 1). No differences in fetal body weight, fetal body length, fetal liver weight, or placental weights were noted (Table 1).

Table 1. *Basic parameters of dams and fetuses on day 19.5 of pregnancy*

	Control Diet	Low Protein
Dams		
Body weight, g	36.5±2.1	35.2±1.3
Liver weight, g	1.6±0.1	1.4±0.1
Ratio of liver weight to body weight, %	4.3±0.3	4.0±0.4
Plasma cholesterol, mmol/l	1.58±0.63	1.17±0.28
No. fetuses	6±3	6±2
Male fetuses		
Placental weight, g	0.110±0.025	0.103±0.016
Fetal weight, g	1.10±0.15	1.20±0.27
Fetal length without tail, mm	20.7±1.7	21.2±2.0
Liver weight, g	0.045±0.007	0.056±0.011
Ratio of liver weight to body weight, %	4.1±0.5	4.4±0.78

Values are means ± SD. Dams were terminated at *day 19.5* ($n = 5$). No. fetuses includes males and females. All fetal characteristics are given for male fetuses only. Fetal length was measured from the forehead to, but not including, the tail. No significant differences were noted.

CpG island microarray identifies 204 differentially methylated gene promoters. To identify CpG islands, which are differentially methylated upon dietary protein restriction and, hence, may be involved in metabolic programming, a 4.6K mouse CpG island microarray was performed on fetal liver DNA. Briefly, unmethylated sequences are digested by methylation-sensitive restriction enzymes, which leads to a relative enrichment of methylated DNA (36). To limit variation, only DNA from male fetuses was used; consequently, only male fetuses were used for all biochemical analyses throughout the entire study.

SAM analysis of the CpG island microarray data revealed 137 clones to be hypermethylated upon protein restriction, and 145 were found to be hypomethylated compared with controls. Because the assay identified all differentially methylated DNA regions, regardless of their position in the proximity of genes, we restricted the analysis to all regions situated closer than 4,000 nucleotides from a transcription start site of known genes. These areas were considered to represent or to contain gene promoters. This restriction yielded 101 regions that were hypomethylated upon dietary protein restriction and 106 regions that were hypermethylated.

After removal of three repetitive sequences, the data were analyzed by the DAVID software package and sorted by biological functions (BP level 4). Of the 183 gene regions that could be annotated (See Supplementary Table A for details. Supplemental data for this article is available online at the *Am J Physiol Regul Integr Comp Physiol* website.), 88 were used for the BP 4 categorization. A total of 36 promoters of genes involved in nucleobase/nucleic acid metabolism was identified. Promoters of genes involved in protein metabolism (33), transport (32), regulation of cellular metabolism (27), and biopolymer metabolism (27) were also identified. Ten differentially methylated regions were linked to precursor/energy metabolism, intracellular transport, establishment of cellular function, or cellular organization, respectively. Finally, four genes involved in heart development were found. Obviously, some genes are involved in more than one pathway.

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Interestingly, the liver-X-receptor alpha (Lxr alpha, Nr1h3) was identified with the CpG island microarray. Lxr alpha is a nuclear receptor that is involved in four of the gene clusters mentioned. The Lxr alpha promoter is contained on clone UHNmmcpg0009844 (http://data.microarrays.ca/cpgmouse/), which was found to be hypermethylated upon dietary protein restriction. Because Lxr alpha is crucial for the regulation of both cholesterol homeostasis and lipogenesis and may, therefore, be involved in metabolic programming, we have chosen to characterize the effects of *Lxra* promoter methylation in more detail.

The Lxra promoter is hypermethylated upon dietary protein restriction. Computer analyses revealed the presence of two short, CG-rich stretches in the promoter of murine *Lxra* (Fig. 1*A*) that are considered as a single CpG island. The first region, with seven CG dinucleotides, ranges from −60 to +6 and, hence, includes the transcription start site. It contains one putative specificity factor-1 (SP1) and two putative MYC-associated zinc finger protein (MAZ) binding sites as postulated by Alberti et al. (1). The second region spans from +117 to +231 and contains the alternative splicing site 1B. In this region, 10 CG dinucleotides can be found.

To characterize the methylation pattern identified in the CpG island microarray on the molecular level, we performed bisulfite sequencing of the *Lxra* promoter CpG island (Fig. 1*B* and Supplemental Table B). In fetuses from dams fed control diet, the CpG island was only marginally methylated. The overall methylation in the protein-restricted group was significantly higher than in the control group (10% upon protein restriction vs. 1% in control; $P < 0.001$). Interestingly, methylation of some CpG position was not effected, while other positions were methylated up to 39% (Fig. 1*B*).

Lxra is regulated by methylation of its CpG island. To assess whether the *Lxra* promoter CpG island indeed regulates expression of the *Lxra* gene, the mouse hepatoma cell line Hepa1.6 was treated with the DNA methylation inhibitor zebularine. Bisulfite sequencing of DNA obtained from control cells and cells treated with 200 μM zebularine (the highest dose tested) confirmed that zebularine blocked methylation of the Lxra promoter CpG island. The average CpG methylation level was reduced from 69% in untreated cells to 17% in cells treated with 200 μM zebularine (see Supplemental Fig. A). In parallel, inhibition of DNA methylation by zebularine led to an increase of *Lxra* mRNA expression in a dose-dependent manner (+320% with 200 μM zebularine) as depicted in Fig. 2.

Zebularine treatment does not specifically act on the *Lxra* CpG island but interferes with DNA methylation in general. To specifically determine the function of the *Lxra* CpG island in vitro, a luciferase reporter assay was performed. For this purpose, the *Lxra* CpG island was cloned into a CpG-free luciferase vector (18) and methylated by *Sss* methylase in vitro. When transfected in the kidney cell line COS7, the activity of the unmethylated *Lxra* promoter construct was 24-fold higher than that of the *Sss*-methylated vector (Fig. 3*A*). As a control, a CpG-free CMV-luciferase construct (18) was treated in the same way and did not show differences in activity (Fig. 3*B*). The experiment was repeated in HepG2 cells and yielded comparable results, a reduction of activity by 97% upon *SSS* methylation of the *Lxra* promoter (data not shown).

LXR target genes are downregulated in the fetal liver upon maternal protein restriction. To study the influence of *Lxra* promoter methylation in vivo, gene expression of *Lxra* and selected LXR target genes were measured in fetal liver at *day 19.5* (n = 8–10). As shown in Table 2, expression of *Lxra* in

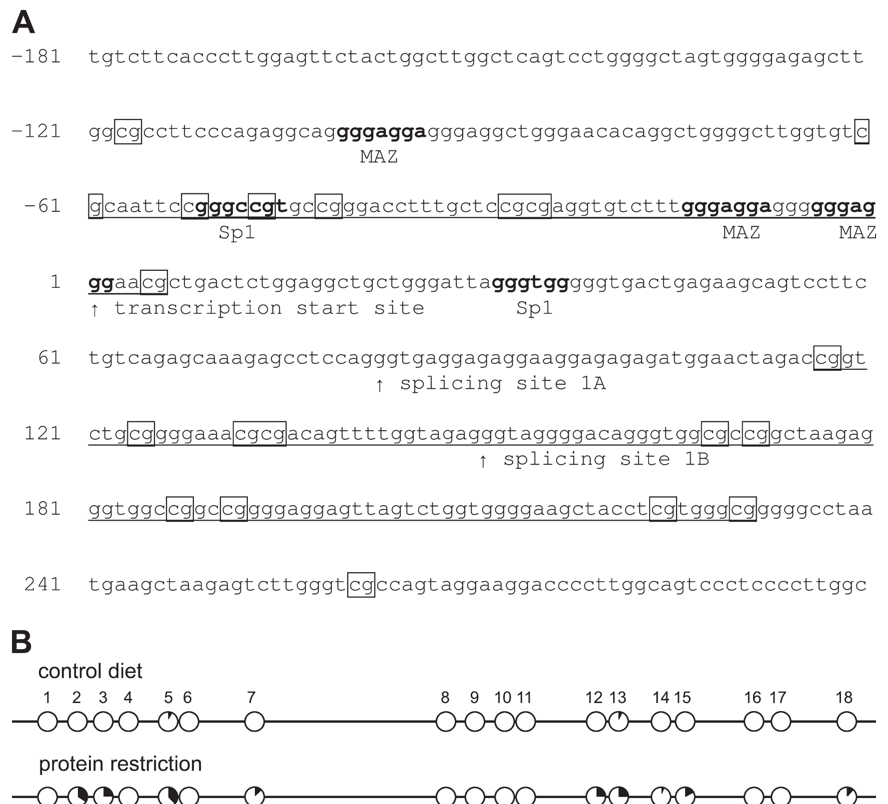

Fig. 1. Methylation pattern of the *Lxra* CpG island. *A*: structure of the murine *Lxra* promoter. Postulated CpG islands are underlined. Transcription factor binding sites according to Alberti et al. (1) are in bold. *Lxra*, liver X-receptor; CpG islands, CG-dinucleotide-rich regions in the promoter of a gene; MAZ, MYC-associated zinc finger protein; SP1, specificity factor 1. *B*: bisulfite sequencing of the *Lxra* promoter. Circles are the CpG positions identified in the *Lxra* promoter. The black inlay is the level of methylation of each CpG position. *Above*, control (15 clones analyzed); *below*, protein-restriction group (18 clones analyzed). A detailed analysis is given in Supplemental Table B.

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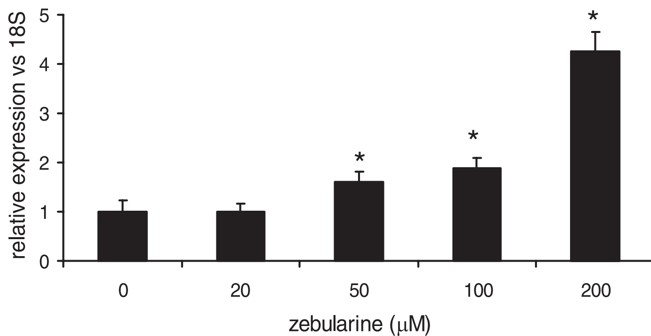

Fig. 2. *Lxra* gene expression in mouse Hepa1.6 cells treated with the methylation inhibitor zebularine. Hepa1.6 cells were incubated for 72 h with (in μM): 0, 20, 50, 100, or 200 of the DNA methylation inhibitor zebularine as described in MATERIALS AND METHODS. *Lxra* gene expression was determined by Taqman real-time PCR. *$P < 0.05$ (Mann-Whitney *U*-test) treated vs. untreated control. Please compare with Supplemental Fig. A for DNA methylation data.

the protein-restricted group was reduced by 32% compared with the control group ($P = 0.03$). Expression of *Lxrb* was identical in both groups (1.00 ± 0.22 vs. 0.91 ± 0.15 in the control and the protein-restricted groups, respectively). In parallel with the reduced expression levels of *Lxra* itself, its target genes showed markedly decreased expression levels in the protein-restricted group compared with controls. Expression of the sterol half transporters *Abcg5* and *Abcg8* was reduced to 44 and 49% of control values, respectively, upon protein restriction ($P = 0.001$ and $P = 0.016$ for *Abcg5* and *Abcg8*, respectively). Expression of *Abca1* was reduced by 18% ($P = 0.027$). Moreover, expression of genes involved in de novo lipogenesis was strongly reduced by protein restriction: *Acc1* expression was suppressed by 28% ($P = 0.001$), *Acc2* by 51% ($P = 0.004$), and *Scd1* by 40% ($P = 0.001$). The gene encoding fatty acid synthase (*Fasn*) was expressed at 76% of control levels that did not reach statistical significance ($P = 0.360$). Expression of the genes encoding the sterol regulatory element-binding proteins (*Srebp1a*, *Srebp1c*, and *Srebp2*) was differentially affected. While the expression levels of *Srebp1a* and *Srebp2* were reduced in the protein-restricted group (by 21 and 24%, respectively), no difference was found for *Srebp1c*. Similarly, no influence of dietary protein restriction on the expression of *Ppara*, *Pparg*, or *Chrebp* was found.

Table 2. *Hepatic gene expression in male fetuses from dams fed control or protein restricted diet*

Genes	Control Diet	Low Protein
Lxra (*Nr1 h3*)	1.00±0.21	0.68±0.19*
Lxrb (*Nr1 h2*)	1.00±0.22	0.91±0.15
Srebp1a	1.00±0.18	0.79±0.19*
Srebp1c	1.00±0.38	0.96±0.12
Srebp2	1.00±0.11	0.76±0.23*
Chrebp (*Mlxipl*)	1.00±0.57	1.03±0.41
Ppara (*Nr1c1*)	1.00±0.18	0.79±0.22
Pparg (*Nr1c3*)	1.00±0.46	0.77±0.19
Abcg5	1.00±0.34	0.44±0.14*
Abcg8	1.00±0.56	0.49±0.18*
Abca1	1.00±0.16	0.82±0.12*
Acc1 (*Acaca*)	1.00±0.12	0.72±0.18*
Acc2 (*Acacb*)	1.00±0.33	0.49±0.31*
Fasn	1.00±0.47	0.76±0.20
Scd1	1.00±0.43	0.60±0.14*
Acadm	1.00±0.26	0.97±0.30
Dnmt1	1.00±0.28	1.08±0.09
Dnmt3a	1.00±0.20	0.87±0.19
Dnmt3b	1.00±0.12	0.93±0.30
Apobec1	1.00±0.21	0.76±0.19*

Values are means ± SD ($n = 8–10$). Hepatic RNA was isolated from male fetuses at *day 19.5* of gestation. Gene expression was studied by Taqman real-time PCR as described in MATERIALS AND METHODS. *$P < 0.05$ (Mann-Whitney *U*-test).

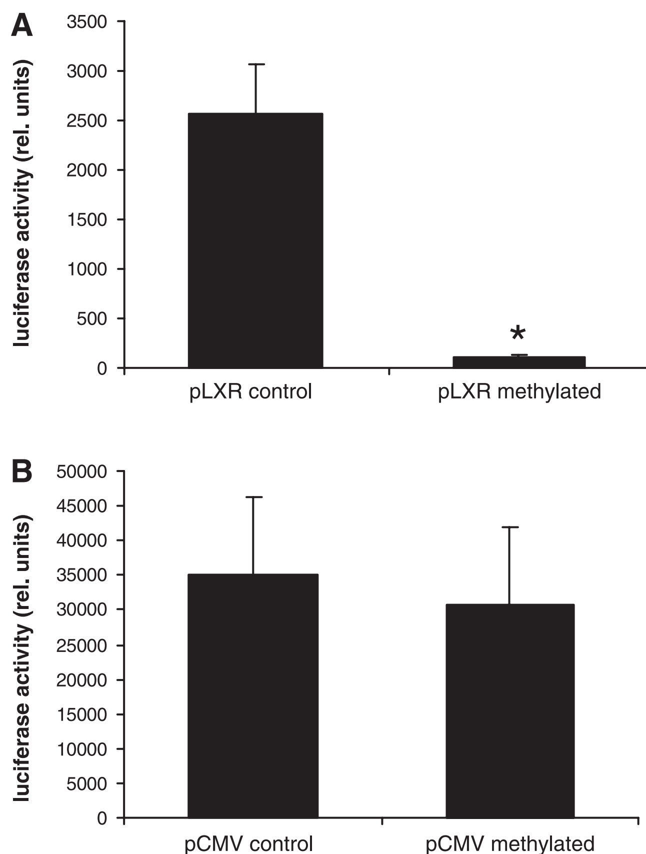

Fig. 3. Effects of promoter methylation on reporter gene expression. *A*: region of the *LXRa* promoter from −926 to +413 was amplified by PCR and cloned into the promoterless, CG-free luciferase vector pCpGl-basic (18). The vector was methylated and transfected in COS7 cells as described in MATERIALS AND METHODS. Luciferase activity was determined 48 h after transfection. Luciferase was normalized to B-galactosidase activity. *$P < 0.05$ (Mann-Whitney *U*-test). *B*: CG-free CMV-luciferase vector pCMV-CpGl was used as control as described before (18). pCMV, cytomegalovirus-promoter-luciferase vector.

To exclude a compensatory overexpression of genes responsible for DNA methylation, the mRNA expression of the genes encoding DNA methyltransferase-1, -3a, and -3b was measured and found to be identically expressed in the protein-restricted group (92%, 102%, and 98% for *Dnmt1*, *Dnmt3a*, and *Dnmt3b*, respectively) compared with control. Expression of the RNA deaminase *Apobec1* was reduced by 24% ($P = 0.021$) in the protein-restricted group, in accordance with the hypermethylation of its promoter region as identified in the CpG island microarray (see Supplementary Table A).

LXR target genes are downregulated in the fetal intestine upon maternal protein restriction. *Lxra* is mainly expressed in the liver and intestine and in macrophages in adult mice. Therefore, we questioned whether the consequences of maternal protein restriction observed in the fetal liver, i.e., down-

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regulation of Lxr target genes, would also be present in the fetal intestine.

We measured expression of *Lxra*, *Lxrb*, and several well-known intestinal target genes of LXR (Table 3). The mRNA expression of both *Lxra* and *Lxrb* was significantly reduced upon maternal dietary protein restriction (by 16% and 34%, respectively). In parallel, the expression of the LXR target genes *Abcg5* and *Abca1* was significantly reduced (by 28% and 39%, respectively). The expression levels of *Abcg8* were very variable between the samples. Therefore, we could not detect a statistically significant effect of maternal protein restriction on *Abcg8* mRNA expression.

Ultimately, the mRNA levels of the DNA methyltransferases *Dnmt1* and *Dnmt3b* were not significantly affected by maternal protein restriction, whereas that of *Dnmt3a* was slightly, but significantly, reduced (−21%) upon maternal protein restriction (Table 3).

Maternal protein restriction interferes with fetal lipid metabolism. Lxr plays a major role in the regulation of hepatic lipid metabolism via regulation of cellular cholesterol homeostasis and de novo lipogenesis. Table 4 shows plasma and liver concentrations of cholesterol, cholestanol, lathosterol, and the two major plant sterols: campesterol and sitosterol. In adult animals, cholestanol and plant sterols are used as surrogate markers for cholesterol absorption, whereas lathosterol levels reflect cholesterol synthesis. In fetal plasma, concentrations of cholestanol and campesterol were significantly reduced upon protein restriction (by 24 and 36%, respectively), whereas total cholesterol, sitosterol, and lathosterol were unchanged. In the fetal liver, cholesterol, and cholestanol concentrations were significantly reduced in the protein-restricted fetuses compared with control-fed animals by 11% and 27%, respectively. No differences were found regarding lathosterol, campesterol, or sitosterol concentrations. Moreover, we could not detect differences in the hepatic concentrations of the common oxysterols 7α-hydroxycholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol (data not shown).

DISCUSSION

Dietary protein restriction during pregnancy is a widely accepted model to mimic fetal undernutrition. It has been shown that dietary protein restriction during fetal development has long-term consequences on many physiological parameters in rodents, an effect commonly referred to as metabolic programming. To explain metabolic programming, morphological changes as well as epigenetic effects, i.e., DNA methylation and histone modifications are currently discussed. Using fetal hepatic DNA, Lillycrop and colleagues (22, 23) have shown that dietary protein restriction leads to hypomethylation of CpG islands in the promoter regions of selected genes. They therefore proposed that a shortage of methyl group donors, together with adaptations in Dnmt1 activity, could lead to global DNA hypomethylation, which would be one possible explanation of metabolic programming. Consequently, restriction of vitamin B12, folate, and methionine lead to global DNA hypomethylation in fetal sheep as identified by restriction landmark genome scanning (29).

Despite the fact that dietary protein restriction is a commonly applied model, genome-wide data on its impact on fetal DNA methylation are not available. We therefore applied a 4.6K mouse CpG island microarray to study differential methylation in a genome-wide way. The genes previously identified to be hypomethylated upon protein restriction, i.e., *GR* and the *PPARs* (22, 23), were not represented with suitable clones on these microarrays. We show here that mild protein restriction of the dam, i.e., in the absence of growth retardation of the pups, leads to hypomethylation of 101 gene-related CpG islands in the fetal liver. Interestingly, 106 regions were hypermethylated. Based on the hypothesis that the previously reported hypomethylation of selected promoters upon protein restriction is a consequence of limited methyl group availability, one would anticipate that hypomethylation would be the major consequence. We could not observe any compensatory upregulation of *Dnmt1*, *Dnmt3a*, or *Dnmt3b*. Our results indicate that the differences in DNA methylation as a consequence of maternal protein restriction may be more a relocation of resources than a passive process caused by substrate shortage. It can hence be hypothesized that maternal protein restriction could, at the molecular level, be mechanistically different from maternal methyl-group limitation as used by Sinclair et al. (29) in sheep.

The CpG islands identified as differentially methylated belong to genes that can be assigned to a variety of biological functions when analyzed by the DAVID software package (10). We focused on *Lxra* (*Nr1h3*), which encodes for a key regulator of cellular lipid homeostasis. In general, *Lxra* is

Table 3. *Intestinal gene expression in male fetuses from dams fed control or protein-restricted diet*

Genes	Control Diet	Low Protein
Lxra (*Nr1 h3*)	1.00±0.16	0.84±0.13*
Lxrb (*Nr1 h2*)	1.00±0.20	0.66±0.15*
Abcg5	1.00±0.24	0.72±0.26*
Abcg8	1.00±0.25	0.79±0.31
Abca1	1.00±0.13	0.61±0.15*
Dnmt1	1.00±0.19	0.84±0.17
Dnmt3a	1.00±0.20	0.79±0.14*
Dnmt3b	1.00±0.25	0.80±0.14

Values are means ± SD (n = 9–10). Intestinal RNA was isolated from male fetuses at *day 19.5* of gestation. Gene expression was studied by Taqman real-time PCR as described in MATERIALS AND METHODS. *P < 0.05 (Mann-Whitney U-test).

Table 4. *Plasma and liver sterol concentrations in male fetuses from dams fed control or protein-restricted diet*

Concentrations	Control Diet	Low Protein
Plasma		
Cholesterol, mmol/l	1.64±0.14	1.39±0.27
Cholestanol, μmol/l	6.95±0.72	5.28±0.96*
Lathosterol, μmol/l	3.18±0.98	3.40±0.77
Campesterol, μmol/l	23.49±5.69	15.10±4.25*
Sitosterol, μmol/l	12.78±3.02	10.53±2.23
Liver		
Cholesterol, μmol/g	25.3±0.96	22.5±0.91*
Cholestanol, nmol/g	73.6±8.92	54.0±5.98*
Lathosterol, nmol/g	107±7.36	122±21.2
Campesterol, nmol/g	184±31.8	139±20.9
Sitosterol, nmol/g	66.2±14.9	54.9±9.36

Values are means ± SD of 5 dams per group and pooled fetal values per group. Sterols were isolated from male fetuses at *day 19.5* of gestation and analyzed by GC-MS as described in MATERIALS AND METHODS. *P < 0.05 (Mann-Whitney U-test).

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highly expressed in the liver, intestine, and macrophages (35). When activated by oxysterols, Lxr alpha binds to its heterodimer partner Rxr. The Lxr/Rxr complex then transactivates expression of genes responsible for removal of cholesterol from the cell (16). This includes genes involved in cholesterol excretion (*Abca1*, *Abcg5/g8*) as well in de novo lipogenesis (*Srebp's*). Thereby, cholesterol is either directly excreted from the cell or can be esterified with newly synthesized fatty acids.

To characterize the influence of CpG island methylation on the expression of *Lxra*, we treated mouse hepatoma cells (Hepa1.6) with the methylation inhibitor zebularine in different concentrations (8). The expression of *Lxra* mRNA increased in a dose-dependent manner. It is tempting to speculate that the demethylation of the Lxra CpG island is responsible for its enhanced transcription. However, as zebularine has promiscuous effects on other genes, which may explain this observation, we used a reporter assay to specifically demonstrate the functionality of the Lxra CpG island. When cloned in front of a luciferase reporter construct (18) and methylated in vitro by *Sss* methylase, the *Lxra* promoter, including the CpG island, almost completely lost its transcriptional function. These findings together demonstrate that the CpG island identified in this paper is able to significantly modify the expression of Lxra, at least in vitro.

The murine *Lxra* promoter contains several transcription factor binding sites, as already described by Alberti et al. (1), namely of the MAZ and SP1. Most interestingly, the first SP1 site (−54 to −49) contains one CG dinocleotide and is surrounded by two others. It has been suggested that cytosine methylation at or close to SP1 motifs is involved in the regulation of SP1 binding to DNA (9). Consequently, SP1 methylation has been shown to occur in a variety of gene promoters, both in normal tissue differentiation and in tumorigenesis. Using bisulfite sequencing, we here demonstrate that the *Lxra* promoter CpG island is indeed selectively hypermethylated upon protein restriction at some positions, while others are not affected. The aforementioned SP1 site belongs to the positions hypermethylated upon protein restriction. It can be hypothesized that methylation of this particular SP1 site, putatively combined with binding of regulatory proteins, is involved in the observed transcriptional silencing of *Lxra* by DNA methylation.

CpG island methylation is generally considered to repress transcription. Consequently, we could observe a small but significant reduction of *Lxra* gene expression in the fetal liver in our protein-restriction model. *Lxra* mRNA expression was 68% ± 19% of control values (P = 0.003; n = 8–10). Downstream, we could observe a major reduction in the expression of a number of well-characterized Lxr target genes, e.g., *Abcg5*, *Abcg8*, and *Abca1*. Abcg5 and Abcg8 together facilitate the transfer of cholesterol and several other sterols from the liver into the bile (6). Unfortunately, we were not able to collect bile from the fetuses. Abca1 is involved in transfer of cellular cholesterol to HDL. Sterol composition of fetal plasma and liver was inconclusive and does not allow an interpretation of the physiological relevance of the observed changes in *Abcg5/g8* expression. This is partially because it is not yet known whether sterol transport mechanisms in adults and fetuses are comparable. Therefore, more work is needed to explore the differences in sterol metabolism observed in our study. On the other hand, LXR has been shown to be involved in the regulation of de novo lipogenesis by regulating the expression of several key genes (17). Consequently, many genes involved in fatty acid metabolism (*Acc1*, *Acc2*, *Fasn*, *Scd1*) were expressed at markedly lower levels in the protein-restricted fetuses. This may have consequences for fatty acid metabolism in the long term.

Besides in the liver, *Lxra* is expressed at high levels also in the intestine and in macrophages. We did not collect fetal macrophages but were able to confirm our finding in the fetal intestine. Similar to the liver, the mRNA expression of the LXR target gene *Abca1* was reduced. Interestingly, mRNA expression of *Abcg5* and *Abcg8* was only mildly (*Abcg5*) or not significantly (*Abcg8*) reduced. Thus, in the liver *Abcg5/Abcg8* are more severely affected than *Abca1*, while in the intestine it is the reverse order. In a very recent paper (33) we have compared the activation of LXR target genes by pharmacological agonists in the fetal liver and the fetal intestine. In that study we found a similar pattern for the upregulation of those transporters, with *Abcg5/Abcg8* being more affected in the liver and *Abca1* in the intestine. This may point to additional factors being involved in the regulation of these genes in the liver and the intestine.

Perspectives and Significance

To the best of our knowledge, we are the first to publish genome-wide methylation data in a model of dietary protein restriction. Our data show that differential methylation of CpG islands may be an important epigenetic regulator of gene expression and hence provide a plausible link between maternal-fetal nutrition and fetal physiology. Despite the fact that we could only detect minor changes in physiological readout parameters in the fetuses, such as sterol concentrations in liver and plasma, it is tempting to speculate that the observed changes in CpG methylation may have long-term consequences on metabolic regulation. This may even be more pronounced when metabolic challenges such as high-fat or high-cholesterol loads require regulatory pathways to come in action.

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