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ORIGINAL ARTICLE

Feeding pregnant rats a low-protein diet alters the hepatic expression of SREBP-1c in their offspring via a glucocorticoid-related mechanism

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Abstract Prenatal exposure to a low-protein diet programmes altered expression of genes that regulate lipid metabolism, including SREBP-1c. The main aim of this study was to investigate whether programmed changes to hepatic SREBP-1c expression in the rat are glucocorticoid-dependent. Rats were fed isocaloric diets (control or low-protein) throughout pregnancy. The low-protein group received 11β-hydroxylase inhibitor, the inhibitor plus corticosterone, or vehicle injections over the first 2 weeks of pregnancy. The control group was administered vehicle injections only. On delivery the animals were transferred to a standard chow diet. The offspring were weaned at 4 weeks of age on to the same chow diet and killed for collection of liver tissue. The inhibitor of glucocorticoid synthesis reversed the suppressive effect of low-protein diet on hepatic SREBP-1c expression of both protein and mRNA seen in low-protein exposed offspring. To test if this effect is through direct effect on the SREBP-1c promoter, H4IIE cells were transfected with a luciferase reporter construct controlled by the SREBP-1c promoter treated with dexamethasone. Dexamethasone induced the expression of SREBP-1c in vitro. Together these studies demonstrate that foetal over-exposure to glucocorticoids, through indirect mechanism, play a crucial role in low-protein-diet-induced changes in lipid metabolism regulating genes.

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Abbreviations

pGL3	pGL3-basic vector; pSREBP1c; SREBP-1c promoter
SREBP-1c	Sterol response element binding protein

Introduction

A broad body of evidence from epidemiological studies suggests that environmental factors encountered before birth, or in early infancy, may exert programming effects upon physiology and metabolism that will manifest as increased disease risk in later life [1, 2]. Low weight at birth or disproportion at birth, particularly when combined with rapid catch-up growth in infancy, are associated with increased risk of coronary heart disease and associated risk factors [3, 4]. The major programming influence operative in foetal life is believed to be maternal undernutrition [5]. Although these epidemiological studies have been criticised, largely on the basis of statistical methodologies [6], animal studies that have considered the impact of prenatal undernutrition upon later health and wellbeing, consistently show that glucose intolerance [7], insulin resistance [8] and hypertension [9] are programmed in utero. Diverse protocols featuring the manipulation of specific nutrients or food restriction during pregnancy [10–14] exert similar long-term programming effects upon the offspring.

Maternal protein restriction is a widely used approach in studies of rat pregnancy [15]. This manipulation of the pregnancy diet generates a phenotype characterised by a brief period of increased insulin sensitivity in early adult

life, followed by declining hepatic glucose handling and insulin resistance as the animals age [16]. It is clear that there are programmed changes in insulin signalling pathways in the liver [17], along with major changes in the expression of transcription factors that regulate lipid metabolism. Studies in our laboratory have shown that intrauterine protein restriction promotes the development of hepatic steatosis in ageing rats due to activation of lipogenesis and suppression of lipid oxidation. These metabolic changes are associated with altered expression of the transcription factors (PPARα, SREBP1c, ChREBP) that regulate lipid-related processes [18]. The phenotype is age-dependent and whilst young adult rats exhibit suppressed lipogenesis and enhanced lipid oxidation, ageing generates a phenotype which favours fat deposition.

The maternal low protein feeding model of rat pregnancy has been widely used to characterise and define the aspects of physiology and metabolism that may be subject to foetal programming. It is proposed that glucocorticoids of maternal origin play an important mechanistic role in this nutritional programming. Maternal low-protein feeding down-regulates the expression of 11β-hydroxysteroid dehydrogenase 2 in the rat placenta [19, 20]. It is suggested that the resulting over-exposure of foetal tissues to maternal glucocorticoids may have a central role in long-term programming of gene expression [21]. The aim of this study was to evaluate whether the suppressive effect of prenatal low-protein feeding on expression of key genes involved in the regulation of hepatic lipid metabolism, in particular de novo lipid synthesis is glucocorticoid-dependent. The main gene of interest was the sterol response element binding protein-1c. SREBP-1c is a transcription factor that activates all genes required for lipogenesis [22]. To supplement this in vivo study we performed a preliminary experiment to assess whether glucocorticoids directly influenced gene expression via the SREBP-1c promoter.

Results

Pharmacological adrenalectomy using metyrapone is a tool that we have used several times previously to explore the role of glucocorticoids in the programming of hypertension and renal angiotensin II receptor expression [23–25]. In this study, 4-week-old offspring of rats fed low-protein diet throughout gestation (group LPV) showed significantly lower expression of SREBP-1c mRNA and protein than control offspring (Figs. 1 and 2). However, the offspring of rats fed the low-protein diet, but treated with metyrapone over the first 14 days of pregnancy (group LPP) exhibited similar SREBP-1c expression to controls. Treatment of rat dams with corticosterone in addition to metyrapone (group LPC) resulted in lower expression.

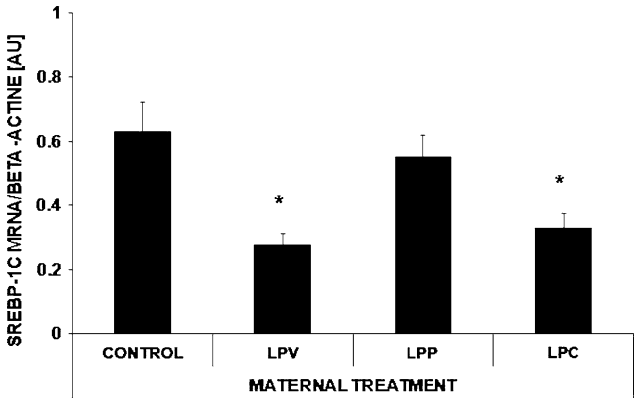

Fig. 1 Expression of SREBP-1c mRNA in livers from the offspring of rats subject to pharmacological adrenalectomy in pregnancy. Pregnant rats were fed control or low-protein diet throughout pregnancy. Control rats and group LPV were injected twice daily with saline vehicle from days 0–14 gestation. Group LPP were administered 5 mg/kg body weight metyrapone twice daily and group LPC were administered 5 mg/kg body weight metyrapone plus 15 mg/kg body weight corticosterone twice daily. Offspring were studied at 4 weeks of age. ANOVA indicated that diet and drug treatment had a significant impact upon hepatic SREBP-1c mRNA expression. * $P < 0.001$ compared to control group. $n = 7$–9 per group

In the in vitro study, H4IIE cells transfected with pSREBP-1c were treated with the synthetic glucocorticoid, dexamethasone. This increased reporter luciferase activity by twofold suggesting that the SREBP-1c promoter in non-LP-exposed samples is responsive to glucocorticoids (Fig. 3), but the nature of the response was markedly different to our observations in vivo.

Discussion

Previous work found that maternal protein restriction leads to down-regulation of 11β-hydroxysteroid dehydrogenase-2 in placenta and consequently, increased foetal exposure to maternal glucocorticoids [19, 20, 25, 26]. This has been proposed to underlie the effects of low-protein diet on offspring phenotypes such as development of hypertension [20, 23]. Glucocorticoid excess has been linked to clinical observations associated with the metabolic syndrome such as central obesity, hypertension, hyperlipidaemia and glucose intolerance [27]. These clinical features associated with glucocorticoids are similar to the phenotypic traits that are programmed in utero following feeding of LP diets in rat pregnancy [9, 18].

SREBP-1c mRNA and protein expression in LP-exposed offspring treated with saline, metyrapone alone (which blocks maternal glucocorticoid synthesis) or metyrapone plus corticosterone were measured to test, in vivo, the dependency of SREBP-1c expression on glucocorticoid-related processes in LP-exposed offspring. The data in this

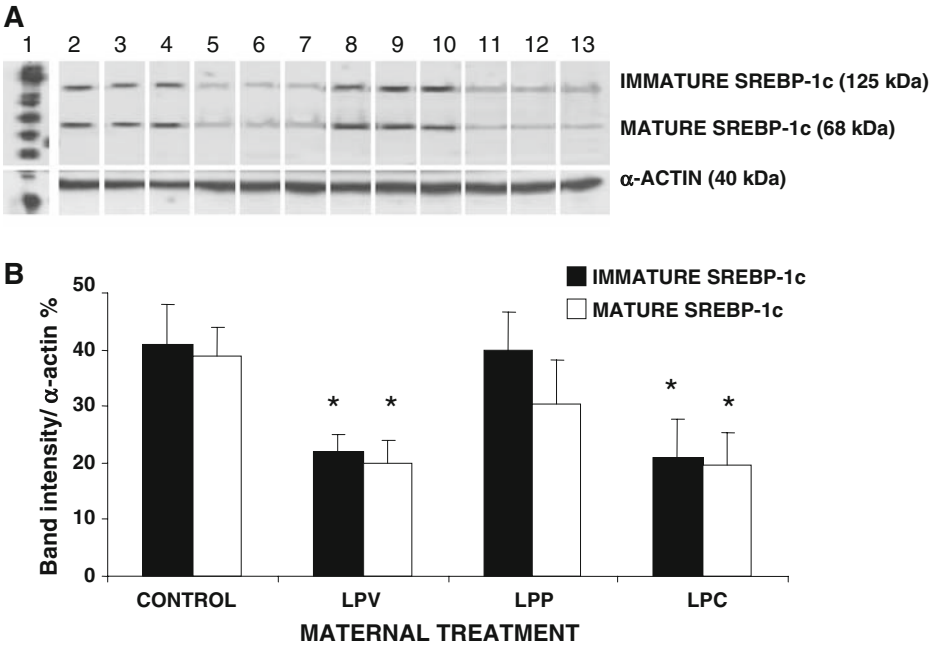

Fig. 2 Pregnant rats were fed control or low-protein diet throughout pregnancy. Control rats and group LPV were injected twice daily with saline vehicle from days 0–14 gestation. Group LPP were administered 5 mg/kg body weight metyrapone twice daily and group LPC were administered 5 mg/kg body weight metyrapone plus 15 mg/kg body weight corticosterone twice daily. Offspring were studied at 4 weeks of age. **a** Western blot analysis of SREBP-1c protein expression in liver tissue. Lane 1 = molecular weight marker, lanes 2–4 = control, lanes 5–7 = LPV, lanes 8–10 = LPP and lanes 11–13 = LPC. α-actin was used for normalisation. **b** Densitometric analysis of SREBP-1c protein expression. Data were analysed using one-way ANOVA with post hoc (Bonferroni Multiple Comparison) test. Values are expressed as mean ± SEM, $n = 6$ per group. (*) indicates significant difference between control and both LPV and LPC groups. P value <0.05

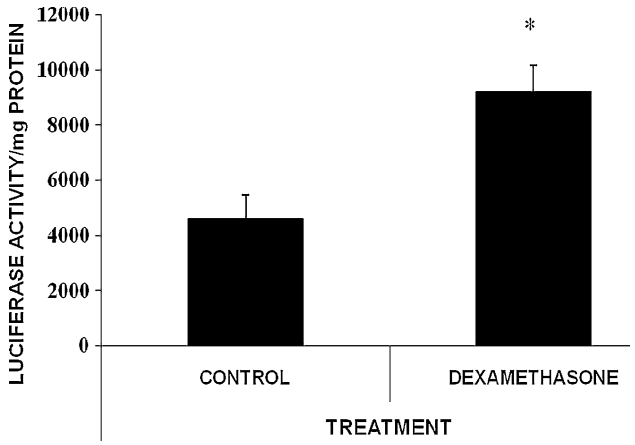

Fig. 3 Impact of dexamethasone treatment upon SREBP-1c expression in transfected cell cultures. Luciferase activity was determined in H4IIE cells transfected with pSREBP-1c. Cells were treated with dexamethasone over 24 h. * $P = 0.005$, compared to the control group. $n = 26$ per group

paper indicate that hepatic SREBP-1c expression was decreased in 4-week-old LP-exposed offspring that received vehicle injection, effectively replicating our previous findings [18, 28]. This study has demonstrated a potential role for glucocorticoids in mediating the effects of prenatal protein restriction upon expression of SREBP-1c. Offspring of rats fed a low-protein diet but subject to pharmacological adrenalectomy (group LPP) did not exhibit the suppressed SREBP-1c mRNA seen in the LPV group. Corticosterone administration reversed this effect. Together these data strongly suggest that manipulation of the materno-foetal exchange of corticosteroids during the first 2 weeks of rat pregnancy has a lasting impact upon expression of hepatic SREBP-1c in later life. Although Woods [29] has recently questioned the relevance of glucocorticoids in mediating nutritional programming, this study and previous work from our and others laboratories [19, 20, 30, 31] provides a weight of evidence in favour of the glucocorticoid programming hypothesis.

The pattern of hepatic SREBP-1c expression noted across the groups in this trial exactly mirrors that which we previously reported for blood pressure and markers of renal morphology in the same animals [23]. These outcomes suggest that glucocorticoids may programme something as fundamental as tissue structure (cell type and number) and hence impact on long-term patterns of gene expression [15, 21]. The low-protein protocol is known to promote remodelling of other organs and systems, including the kidney [32], pancreas [33] and brain [34]. Within the liver, it is possible that prenatal insults modify the metabolic zonation of the liver, with an increase in numbers of cells

in the periportal region and a decrease in the perivenous region [35]. Phosphoenolpyruvate carboxykinase (PEPCK) is a largely periportal enzyme and has been shown to be up-regulated in adult rats following prenatal dexamethasone treatment [36]. This is therefore consistent with a hepatic remodelling action of glucocorticoids in foetal life.

In addition to the main in vivo study, an in vitro (transfection) study was carried out to address two issues. Firstly, prior to this investigation there have been no published reports describing an effect of glucocorticoids on hepatic SREBP-1c expression under normal conditions. It is well established that SREBP-1c expression is regulated by hormones such as insulin and glucagon. However, there were no data about the possible regulation by glucocorticoids. Therefore, to better understand the glucocorticoid response of hepatic SREBP1c in LP-exposed offspring (pre-treated samples) it was essential to know whether glucocorticoids directly modulate the mRNA expression of SREBP-1c. The second aim was to test whether any glucocorticoid mediated effect is through direct interaction between glucocorticoids and the SREBP-1c promoter.

In this study we investigated the possible effect of glucocorticoid hormones on SREBP-1c expression in cultured hepatocytes (H4IIE cell line). Although apparently paradoxical when viewed alongside the in vivo study, the data from this study is of great importance as it demonstrates for the first time that treatment of a cultured cell line with dexamethasone, after transient transfection with pSREBP-1c results in increased SREBP-1c promoter activity. The transfection study was not intended to assess long-term epigenetic reprogramming but simply provides an indicator of whether the SREBP-1c promoter is glucocorticoid responsive.

The in vitro work suggests that glucocorticoids up-regulates SREPB-1c expression, whilst in vivo the opposite effect was observed. It is important to note that the in vivo and in vitro studies were not direct equivalents. In cell culture we examined the immediate impact of a synthetic glucocorticoid upon gene expression. In vivo we examined the effect of blockade of maternal glucocorticoid synthesis upon expression 5 weeks after the cessation of treatment. Any effect of glucocorticoids on SREBP-1c expression could be mediated in a cell or tissue-specific manner. It is obvious from comparing promoter analysis (in vitro) study and in vivo data that glucocorticoids have an effect on SREBP-1c mRNA expression which is not a consequence of direct interaction with the promoter, but is rather a much complex mechanism that need to be further explored. We therefore suggest that programming of SREBP-1c mRNA and protein expression by glucocorticoids in vivo may be through indirect mechanisms, for example alterations in tissue structure, rather than direct effects at the promoter level. Furthermore, metyrapone is not specific to glucocorticoids in its action. Broad disturbances of maternal endocrine function will have followed the administration of this agent and although the inclusion of the corticosterone replacement group was intended to elucidate the glucocorticoid-specific effects of the drug, the observed effects may be due to fluctuation in concentrations of other hormones in maternal and foetal circulation. The LPV group exhibited a suppressed expression of SREBP-1c, as noted in our earlier studies [18, 28]. It is of interest to note that this suppression of expression occurs only after birth (i.e. once the programming stimulus is withdrawn, [28]), which further adds to our assertion that glucocorticoid exposures may exert effects through modification of tissue morphology, resulting in patterns of gene expression that are longer-term adaptive responses to insult.

Conclusion

The findings of this paper provide confirmation that prenatal undernutrition has the capacity to produce perturbations in the expression of transcription factors involved in control of lipid metabolism. Changes in gene expression occur around the time of birth [28] and may be precipitated by over-exposure of the developing foetus to glucocorticoids of maternal origin, or by other events that are induced by such exposures. These findings raise the prospect of interventions with agents that target SREBP-1c as means of counteracting the programming effects of undernutrition.

Materials and methods

In vivo studies

Animals

The experiments in this paper were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were licensed by the Home Office. Animals were held under temperature-controlled conditions on a 12 h light: dark cycle. The animals had ad libitum access to food and water at all times.

Modulation of intrauterine glucocorticoid exposures

The aim of this trial was to assess the role of glucocorticoids as mediators of the programming effect of a low-protein diet in rat pregnancy. Twenty-four virgin female Wistar rats (Harlan Ltd, Belton, Leics, UK) were mated at weights between 200 and 250 g as described above. Upon confirmation of mating the rats were allocated to be fed

control diet ($n = 6$) or low-protein diet ($n = 18$), throughout pregnancy. The 11β-hydroxylase inhibitor metyrapone was used to inhibit the synthesis of corticosterone by the maternal and foetal adrenal glands [37] and thus test the glucocorticoid dependency of LP effects. The LP fed rats received twice daily injections of metyrapone (5 mg/kg body weight; group LPP, $n = 6$), metyrapone plus corticosterone (15 mg/kg body weight; group LPC, $n = 6$) or injection vehicle (saline; group LPV, $n = 6$) over days 1–14 of pregnancy, as reported previously [23]. Control rats received vehicle injections. The main outcomes of this experiment (blood pressure and renal function of the offspring) are reported elsewhere [23]. Previous work has demonstrated that metyrapone does not impact upon tissue development in offspring of rats fed the control diet [24, 25], there was no control diet metyrapone-treated group included in the study. Metyrapone was administered at a dose previously shown to have no adverse effect upon reproductive outcome and to reduce maternal corticosterone concentrations by 90% [24, 25]. The pregnant rats remained on the semi-synthetic diets until they gave birth at 22 days gestation. All animals were then transferred on to standard laboratory chow diet (B&K Universal Ltd, Hull, UK) and the litters culled to eight pups to minimise variation in suckling nutrition. At 4 weeks of age, offspring were weaned onto chow diet and killed by CO_2 asphyxia followed by cervical dislocation. The livers were rapidly excised and frozen in liquid nitrogen before storage at −80°C for later analyses.

Determination of mRNA expression

RNA was isolated using the method described by Chomczynski and Sacchi [38]. cDNA was synthesised using the Taqman Reverse Transcription Reagents kit and then quantitative RT-PCR was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, UK). Fluorogenic probes were labelled with FAM (6-carboxy-fluorescein) at the 5′ end and with TAMRA (6 carboxy-tetramethyl-rhodamine at the 3′ end. A negative template control and relative standard curve were included on every PCR run. The standard curve was prepared from a pool of sample cDNA over a 4-fold range of dilutions. Relative SREBP-1c mRNA quantity was calculated from the standard curve and all samples were normalised against, the house-keeping, eukaryotic 18S rRNA (Applied Biosystems, UK). 18S rRNA expression did not vary significantly between the different experimental groups in the tissue studied. Sequences of SREBP-1c primers and probe used for the PCR studies are shown in Table 1.

Determination of protein expression

The expression of proteins in the liver was quantified using Western blotting, as described previously [18]. Briefly, hepatic protein was extracted using the method of Chomczynski and Sacchi [38]. Isolated protein concentrations were quantified using the method of Lowry et al. [39] adapted for use in a 96-well micro-assay plate. Seventy-five micrograms of protein sample was denatured by boiling for 5 min and samples were then loaded onto an SDS polyacrylamide gel for separation (40 mA for 2 h). Separated proteins were transferred on to Hybond ECL (enhanced chemiluminescence, Amersham-Pharmacia Biotech) nitrocellulose membrane and after blocking of non-specific binding sites, membranes were incubated overnight with primary antibody solutions at 4°C. For SREBP-1c the primary antibody was SREBP-1 specific, developed in mice (ATCC, Middlesex, UK). Expression of α-actin protein was used to normalise specific protein expression. For this a rabbit anti-α actin was used as the primary antibody (Sigma-Aldrich, USA).

After this incubation, further washing and blocking steps were followed by incubation with a horseradish peroxidase-labelled secondary antibody to allow imaging of antibody binding using AIDA Image Analyser software (Raytest, Gmbh). For SREBP-1c the secondary antibody was peroxidase-conjugated rabbit anti-mouse (Dako, Cambridgeshire, UK). For α-actin the secondary antibody was peroxidase-conjugated swine anti-rabbit (Dako, Cambridgeshire, UK).

Transfection studies

In order to assess the effect of glucocorticoids on SREBP-1c gene transcription rate, rat hepatoma cells (H4IIE, ATCC Manassas, USA) were cultured in 6-well plates containing Dulbecco's Modified Eagle medium with 10%

Table 1 Sequences of primers and probes used in PCR

Gene	Sequences 5′–3′	T_m °C	Amplicon (bp)	Size accession no
Eukaryotic 18S rRNA	Supplied by Applied Biosystems (ABS)		190	X_03205
SREBP1c	Forward: GGAGCCATGGATTGCACATT	63.15	191	L_16995
	Reverse: AGGAAGGCTTCCAGAGAGGA	60.47		
	Probe: ACATGCTTCAGCTCATCAACAACCAA	69.28		

(v/v) foetal bovine serum and incubated at 37°C, 5% CO_2 for 2 days. Cultures plated at 2.5×10^5 cells per well were allowed to reach 60–70% confluence and were then transfected with pGL3 basic vector in which the luciferase gene was under the control of the SREBP-1c promoter [40], using polyethyleneimine as described by Thomas and Klibanov [41]. Following transfection the cells were placed in fresh medium and incubated with 1 μM dexamethasone for 24 h. Cells were then harvested, washed and lysed for assay of luciferase activity using Promega luciferase assay reagent. Luciferase activity was expressed as arbitrary luminescence units per mg of cellular protein.

Statistical analysis

All data are presented as mean ± SEM. Unless stated otherwise in the text, data were analysed using one- or two-way analysis of variance using SPSS version 11.5. Where ANOVA indicated a significant effect of treatments, post hoc tests were performed using a Bonferroni test. In all analyses initial ANOVA considered the impact of sex upon the variables studied. No univariate effects of sex or interactions of sex with other factors were noted, and so all data in this paper show measurements for male and female animals combined.

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