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Altered cellular redox status, sirtuin abundance and clock gene expression in a mouse model of developmentally primed NASH☆

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ABSTRACT

Background: We have previously shown that high fat (HF) feeding during pregnancy primes the development of non-alcoholic steatohepatits (NASH) in the adult offspring. However, the underlying mechanisms are unclear.
Aims: Since the endogenous molecular clock can regulate hepatic lipid metabolism, we investigated whether exposure to a HF diet during development could alter hepatic clock gene expression and contribute to NASH onset in later life.
Methods: Female mice were fed either a control (C, 7% kcal fat) or HF (45% kcal fat) diet. Offspring were fed either a C or HF diet resulting in four offspring groups: C/C, C/HF, HF/C and HF/HF. NAFLD progression, cellular redox status, sirtuin expression (*Sirt1*, *Sirt3*), and the expression of core clock genes (*Clock*, *Bmal1*, *Per2*, *Cry2*) and clock-controlled genes involved in lipid metabolism (*Rev-Erbα*, *Rev-Erbβ*, *RORα*, and *Srebp1c*) were measured in offspring livers.
Results: Offspring fed a HF diet developed NAFLD. However HF fed offspring of mothers fed a HF diet developed NASH, coupled with significantly reduced NAD^+/NADH ($p < 0.05$, HF/HF vs C/C), Sirt1 ($p < 0.001$, HF/HF vs C/C), Sirt3 ($p < 0.01$, HF/HF vs C/C), perturbed clock gene expression, and elevated expression of genes involved lipid metabolism, such as Srebp1c ($p < 0.05$, C/HF and HF/HF vs C/C).
Conclusion: Our results suggest that exposure to excess dietary fat during early and post-natal life increases the susceptibility to develop NASH in adulthood, involving altered cellular redox status, reduced sirtuin abundance, and desynchronized clock gene expression.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disease worldwide, and is present in a third of the general population and the majority of individuals with obesity and type 2 diabetes [1,2]. In its milder form, NAFLD is characterized by excessive intra-hepatocyte triglyceride (TG) accumulation (hepatic steatosis). In advanced stages steatosis is coupled with inflammation and termed non-alcoholic steatohepatits (NASH) [3], which can eventually result in liver failure. NAFLD is commonly associated with older individuals; however, recent evidence suggests that NAFLD is increasingly common in young adults, children and adolescents [4–6]. The precise mechanisms underlying the premature onset of liver disease are unclear.

A substantial body of evidence suggests that early life is a critical period of plasticity, in which the developing organism physiologically adapts to its surrounding environment. In some cases this may be advantageous [7,8], however, imbalanced nutrition during early life may also have deleterious effects on the development of key metabolic organs. This is particularly true of the liver, which undergoes important maturation stages during late gestation and early postnatal life. Hence, the liver is highly susceptible to chronic maternal high fat (HF) diets, which can lead to a NAFLD phenotype in offspring independent of maternal and offspring obesity [9]. In addition, we have previously shown in mice, that exposure to a high fat (HF) diet during gestation and lactation primes the development of the severe form of fatty liver (NASH) in adult offspring, involving impaired mitochondrial function

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HF, high fat; C, control; sirt1, sirtuin 1; sirt3, sirtuin 3; Clock, circadian locomotor output cycles kaput; Per2, Bmal1 (also called Arntl1), period 2; Cry2, cryptochrome 2; RORα, retinoic acid receptor-related orphan receptor alpha; Srebp1c, sterol regulatory element binding protein-1c; NAD, nicotinamide adenine dinucleotide; OPN, osteopontin.

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and increased expression of genes involved in lipogenesis [10]. Further studies support the notion that early HF exposure can prime the onset of NAFLD in adulthood. In a rat model of high fat feeding during pregnancy only, obese mothers gave rise to offspring with altered transcriptional and epigenetic regulation of key regulators of fatty acid oxidation such as peroxisome proliferator-activated receptor (PPAR)α [11]. Similarly offspring of HF fed dams have shown increased expression of the lipogenic transcription factor, *Srebp1c* [12,13].

Nutritional status is constantly being assessed by transcriptional networks that control lipid homeostasis [14]. In mammals, one such network is the endogenous molecular clock network, which mediates transcriptional changes controlling a number of metabolic outputs [15]. While the suprachiasmatic nucleus (SCN) of the hypothalamus is the central pacemaker, molecular clocks also exist in peripheral tissues such as the liver where they can be entrained by local nutrient availability [16]. The core components of the clock network *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1 and Cry2*, self-regulate through transcriptional feedback loops resulting in 24-hour oscillatory patterns of gene expression [17,18]. Interestingly, genetic disruption of the clock genes and changes in the canonical 24-hour oscillatory patterns can lead to fatty liver disease [19,20]. This is likely due to the fact that the core clock genes transcriptionally regulate downstream clock controlled genes (CCGs) with important roles in hepatic lipid metabolism. For example, *Bmal1* has been shown to directly regulate CCGs involved in hepatic carbohydrate and lipid metabolism [21]. In addition, the *Rev-Erb and ROR* nuclear transcription factors are able to activate and repress *Bmal1* expression respectively, thus fine tuning the circadian regulation of metabolic genes [22]. The *Rev-Erb* isoforms, previously considered as accessory components of the clock system, have been shown to coordinate a number of genes involved in lipid metabolism [23]. Recent studies using *Rev-Erb* agonists have revealed alterations in the nycthemeral (day vs night) patterns in both behaviour and hepatic gene expression [24]. Importantly, the maternal environment has the capacity to alter clock gene expression in the developing liver, causing changes that persist postpartum [25]. For example, in non-human primate liver, exposure to a maternal high-fat diet significantly alters expression of fetal hepatic *Npas2*, a paralog of the clock transcription factor [26]. In rodents, offspring of obese dams show reduced hepatic expression of both circadian and metabolic genes, which is associated with altered mRNA dynamics [11]. More recently it has been shown that maternal obesity interacts with an obesogenic post-weaning diet to disrupt the canonical rhythmicity of gene expression in the liver, and alters the DNA methylation levels at both the *Bmal1* and *Per2* promoters [27].

Recent findings suggest that the *Clock* and *Bmal1* heterodimer sense the metabolic status of the cell via a functional interaction with the NAD^+ dependent deacetylase SIRT1 [28,29]. SIRT1 belongs to a class of sirtuin proteins with roles in circadian rhythms, mitochondrial metabolism, and aging [30–32]. Moreover, recent findings have shown that SIRT3, a mitochondrial protein deacetylase, is down-regulated in response to maternal fat exposure and is involved in the development of fatty liver disease [33,34]. Thus, it is possible that SIRT1 and SIRT3 functionally link mitochondrial metabolism, lipid homeostasis, circadian biology and age-associated metabolic decline.

In light of these findings we tested the hypothesis that HF feeding during development could contribute to the priming of severe fatty liver disease, through altered circadian biology, clock gene expression, and altered regulation of CCGs with key roles in lipid homeostasis.

2. Methods

2.1. Animal model

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the local ethics review committee. Female C57/BL6J mice were maintained under a 12-hour light/dark cycle (lights on at 07.00 h), and at a constant temperature of 22 ± 2 °C with food and water available ad libitum. Dams were randomly allocated to one of two diets: control (C; 7% kcal fat; Special Dietary Services, UK), or a high fat diet (HF; 45% kcal fat; Special Dietary Services, UK). We have previously used this HF diet to bring about an obese phenotype in both the pregnant dams and their offspring [10]. Dams were fed the designated diet 6 weeks pre-pregnancy, through to pregnancy and lactation. Pregnant dams were allowed to deliver their pups, and litter size was standardized to six pups (three males and three females, whenever possible) to ensure that no litter was nutritionally biased. Offspring were randomly allocated to either the C or HF diet at weaning at 3 weeks of age resulting in 4 offspring groups; C/C, C/HF, HF/C and HF/HF. These offspring were fed the diets for the next 12 weeks. At 15 weeks of age, male offspring were killed by cervical dislocation at 2 time points during the 24-hour light-dark period, at 3 pm (Zeitgeber time 8 or ZT8; 8 h into the light or day period) and at 3 am (ZT20; 8 h into the dark or night period). Liver tissue was immediately frozen in liquid nitrogen and stored at −80 °C or paraffin embedded for histological analysis.

2.2. Day-night changes in food intake and energy expenditure

Food intake and energy expenditure were recorded over a 24-hour light–dark cycle in the four offspring groups (C/C, C/HF, HF/C and HF/HF, n = 6–10 per offspring group) using a closed modular indirect calorimetric system at 12 weeks of age (Oxylet, Panlab SLU, Spain). Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were recorded at 5-min intervals using a computer-assisted data acquisition program (Metabolism; Panlab SLU, Spain) over a 24-hour period. From this, the animal's energy expenditure (EE; in kcal/day/body $weight^{0.75}$) was calculated. Food intake was continuously measured using an extensiometric food weight transducer device (Panlab SLU, Spain). To determine day and night differences in food intake and energy expenditure between the offspring groups, the mean values were calculated during the light and dark phases of the cycle for each mouse in each treatment groups.

2.3. Glucose tolerance test

Glucose tolerance test was performed after an overnight fast at 14 weeks of age. Fasting glucose concentration was measured in whole blood obtained from the tail vein before the fasted mice were intraperitoneally injected with D-glucose (2 g/kg mouse body weight), and blood glucose concentration was measured using a glucometer (Aviva Accu-Chek, Roche Diagnostics Ltd., UK) at 15, 30, 60 and 120 min.

2.4. Evaluation of steatosis by point counting

Evaluation of steatosis was done by point counting. Previous studies have shown that the point counting method of evaluating steatosis had a strong and significant correlation to hepatic triglyceride levels [53]. Briefly, captured digital images of liver sections stained with hematoxylin and eosin (H&E) were projected on an LCD monitor. For each H&E stained liver sections, sequential images were taken at 20× magnification across a section (avoiding large vessels). A total of 3 stained sections were scanned per animal (n = 4 animals per treatment group). Using a computer software FIJI (http://fiji.sc/Fiji), a 10 × 10 grid system of 100 test points (PT) was superimposed on the image field. The percentage volume density of hepatic steatosis (Vv[steatosis, liver]%) was then estimated as the ratio of the points hitting the vesicles of fat (Pp) compared to the number of test points: Vv[steatosis, liver]% = (Pp[steatosis] / PT) × 100.

2.5. Histological analysis

The Kleiner scoring system was used to assess the severity of NAFLD [35]. An activity score (AF) was generated by adding the individual