FOOD-CT-2005-007036

EARNEST

EARly Nutrition programming- long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research

Instrument: Integrated Project

Thematic Priority 5.4.3.1: Food Quality and Safety

Final public report on activity 3.1.3:

Impact of genotype on programming responses to protein supplementation: cardiovascular control and appetite

Period covered from 15.04.2005 to 14.10.2010

Start date of project: 15.04.2005 Duration: 5,5 Years

Organisation Name of Lead Contractor for this report: FBN

High-protein (HP) diets have been shown to cause weight loss by inducing longer postprandial satiety followed by lower subsequent food intake, and increasing dietary thermogenesis. In obese and diabetic people consuming diets high in protein, a reduction of body fat tissue and improved glucose homeostasis was reported. Similar results were obtained in rats fed HP diets with reduced carbohydrate content. Thus, due to its metabolic effects helping to control body mass gain high protein diets are popular and may be consumed also in women in childbearing age even before they consciously know they are pregnant and during pregnancy.
Reports on the impact of an increased dietary protein intake during pregnancy and lactation on maternal and offspring health and development are scarce. However, there seems to be a similarity with the effects of a low-protein diet that also causes decreased birth weight and altered body mass (BM) development of the offspring during infancy. Epidemiological studies in women show that high protein intakes during pregnancy also resulted in growth retardation of the baby. Excess of nutrients in reproducing females during pregnancy and lactation can lead to altered milk production and milk composition, due to alterations in mammary gland (MG) structure and gene expression. Thus, high protein diets may also affect early life nutrition of infants when nourished by mother's milk exclusively.
Leucine is a branched chain amino acid and is known to be involved in regulation of protein synthesis (translational control), insulin action, lipogenesis and fuel utilization in muscle. Leucine is especially rich in dietary proteins of dairy food products. We hypothesized that some of the effects of a high protein diet in pregnant and lactating females might be due to the high content of dietary leucine. Thus, the effect of a diet being equivalent in protein content to an adequate control diet but high in leucine comparable to the leucine content in a high protein diet was evaluated in the offspring of pregnant and lactating mouse dams.
It is known that different genotypes behave differently in their reaction to different diets. We were therefore interested to know whether this is the case in regard to a high protein or a high leucine diet. A further aim was therefore to investigate whether a maternal high protein or high leucine diet during pregnancy and lactation would affect growth and development of the offspring of various genotypes.

The initial mouse population was derived from a crossing of four outbred (NMRI orig., Han:NMRI, CFW, CF1) and four inbred (CBA/Bln, AB/Bln, C57BL/Bln, XVII/Bln) populations. The unselected control mouse line (CON) was bred by random mating for 125 generations. The mouse line long-term selected for high body weight (HBW) was constructed by sib selection of the heaviest males per litter at age 42 days for 126 generations, whereas the LDR mouse line was bred by selection of males with the longest running distance covered at age 70 d for 92 generations.

The specific objectives were, to

1. study the effects of a high protein diet during pregnancy and /or lactation in mice of three different genotypes ((HBW, LDR, CON) on pregnancy outcome and offspring growth, development, and energy and macronutrient metabolism,
2. study the effects of a high leucine diet during pregnancy and /or lactation in mice of three different genotypes (HBW, LDR, CON) on pregnancy outcome and offspring growth and development,
3. study maternal growth and development as well as lactation parameters and structural or functional features of the mammary gland in two mouse genotypes (HBW, CON) when fed a high protein during pregnancy and lactation.
4. study gene and proteome expression in the liver of offspring and pregnant and lactating dams, respectively, to identify potentially responsible mechanisms.

Three experiments were performed.
Experiment A: Female mice of 3 strains (CON, HBW, LDR) were fed isoenergetic HP (40% protein) or control (C) diets (20%) from mating to end of lactation (21d). Litters were standardized to 10 pups at birth. Pups were cross-fostered and offspring was tested in 3 combinations of pre- and postnatal dietary exposure: HP-C (prenatal-postnatal), C-HP, and C-C (n=10 litters each). After weaning all

pups were fed normal diets. Growth and body composition was monitored until age 400 d. Subsets of offspring were killed at 22, 42, 160 and 370 d of age to sample tissues and plasma. Indirect calorimetry measurements were performed at age 22, 42, 160, and 360 d. Muscle characteristics were analyzed at age 23 and 180 d.
Experiment B: A similar experimental design was performed as in Experiment A with the exception that the effects of a pre- or early postnatal exposure to a high leucine (HLeu) diet were investigated. The comparison of three treatments in offspring was performed: C-C, HLeu-C, C-HLeu. After weaning all pups were fed normal diets. Growth and body composition was monitored until age 400 d. Sample collection and analysis was performed as described above.
Experiment C: Aim: To explore reasons for the reduced growth rate of offspring born to dams fed C diet during pregnancy but HP diet during lactation. Maternal body mass and body composition development, mammary gland development (mass and histology) and gene expression, as well as milk composition, plasma metabolites, and hepatic proteome profile during pregnancy and lactation were analysed, respectively. Dams were fed C and HP diet throughout pregnancy and lactation (C-C, HP-HP) or switched to the alternative diet at day 1 after parturition (HP-C, C-HP). Experiment C was performed in strain CON and HBW only.

Growth and body composition
In HBW, CON, and LDR maternal growth from mating to term was reduced by 64, 45 and 11% in HP fed dams as compared to C dams (HBW, CON; $P<0.05$). We could not find differences in food intake among diets. CON and LDR offspring showed reduced birth weight when their mothers received HP diet in pregnancy (CON 1.40 vs. 1.46 g; LDR 1.58 vs. 1.62 g; $P< 0.05$). HBW offspring showed no differences (2.17 vs. 2.12 g). Litter size at birth was lower in CON dams fed HP diet (11 vs.12 pups with C diet; $P< 0.05$). During lactation food intake (foster dam+litter) in foster dam did not differ within lines with exception of HBW where HP foster dams reduced the food intake by 30% ($P<0.05$). Until weaning, losses were highest among C-HP pups (CON 23% and HBW 16% of pups among all litters). In all strains body mass gain per litter between birth and age 21d were lowest in C-HP (CON 43.9; LDR 51.8; HBW 88.1 g), as compared to HP-C (95.1; 71.7; 199.9 g) and C-C (95.7; 78.7; 197.3g) offspring (lactation diets HP vs. C; $P<0.05$). In the CON and the LDR line the body length (nose - anus) at weaning age (d 21) was lower in C-HP as compared to C-C offspring (CON: 7.63 vs. 8.64 cm; LDR: 7.04 vs. 7.73; $P< 0.05$). At age 42 d HP-C offspring of the CON line was shorter than the control C-C (9.53 vs. 9.81 cm, $P< 0.10$) whereas no difference between C-HP and C-C was observed. In contrast, at age 42 d in the LDR line body length was not different among treatments.

In all 3 genotypes body mass development until age d 95 was lowest in C offspring reared by HP foster mothers ($P< 0.05$) with no difference in body mass between offspring groups C-C and HP-C. In C-HP offspring growth was reduced until age d 28 and catch-up growth occurred between age d 35 – 49 ($P< 0.05$). In the CON line body mass was lower in the C-HP group until age 175 d ($P< 0.05$). This difference disappeared thereafter and body mass did not differ among C-C and HP-C offspring until age 400 d. In the HBW line offspring from the C-HP treatment group remained lightest in body mass until age 275 d. These differences disappeared at age 370 d. In HBW offspring suckled by foster mothers fed HP diet (C-HP) also body length tended to be reduced ($P<0.10$). Food intake in offspring was monitored between age 35-42, 133-140, and 350-357 d. No treatment-dependent differences in food intake were observed among CON and LDR offspring groups. In HBW the C-HP group had a lower food intake between age 35-42 (10.5 g/d), and 133-140 d (11.0 g/d) as compared to C-C (12.4 and 11.5 g/d) and HP-C (12.2 and 11.7 g/d) offspring ($P<0.05$).

No difference in body fat% between dietary treatments (C-C, HP-C, C-HP) at age 21 and 42 d were found in CON. In LDR the body fat % showed a tendency to be lower in HP-C at age d 42 ($P<0.12$). At age 160 d the C-HP offspring group had a tendency ($P<0.12$) for less body fat in CON and LDR, and was leaner in HBW ($P<0.05$). In the LDR offspring group HP-C a lower body fat content at age 370 d was noted ($P<0.05$). In contrast to the CON and LDR lines the HBW genotype lost body fat from age 160 to 370 d which was most distinct in animals which were born to mothers fed high protein diet during pregnancy. This decrease in body fat% was confirmed by the decrease in weight of

gonadal fat depots. Age-dependent body fat mass increase in the LDR line was less in offspring exposed to maternal high protein diets in utero (HP-C).

We conclude that maternal HP diet during pregnancy in mice reduces birth mass in a genotype dependent fashion. Pups suckled by HP dams grow less until weaning and thereafter. This appears to be partly related to a lower body mass gain in dams fed HP. The HBW offspring consumed less food during adolescence and young adulthood. The LDR strain turned out to be less susceptible to pre- and early postnatal exposure to maternal HP diets.
A high maternal leucine intake during pregnancy or lactation did not affect birth mass, body mass, body length and body fatness and food intake of the offspring up to about age 1 year in the LDR and HBW lines. In contrast, in the line CON high maternal leucine intake during pregnancy or lactation was associated with a small but significantly higher offspring body mass between age 63 d and 225 d. The C-HLeu CON offspring had a higher body mass until age 300 d. This difference disappeared thereafter. We therefore conclude that the persistent growth retardation observed in offspring reared by foster mothers fed diets containing HP diets (C-HP), which was line dependent, could not be explained by a high leucine intake but rather by a high amino acid (protein, resp. nitrogen) intake per se and/or the lower carbohydrate content in the HP diets which probably provided less net energy to gestating and lactating mothers with consequence for lactogenesis in the mothers (see below).

Muscle characteristics
We have analysed muscle mass/characteristics and enzyme activities (lactate dehydrogenase, isocitrate dehydrogenase, creatine kinase) of *musculus rectus femoris* shortly after weaning (23 d) and at age 180 d. At 23 d, offspring exposed to a maternal HP diet during suckling experienced a reduced muscle growth whereas possibly negative effects due to exposure to a HP diet in utero (lower birth weight as observed earlier) can be ameliorated by rearing at a control dam.
Overall, the effect early life HP maternal diet exposure in 180 d old mice seemed small as compared to the genotype effect with the LDR genotype showing the lightest and the HBW the heaviest muscle. Muscle protein concentration was numerically highest in LDR. Generally, the HBW line had a more active glycolytic and oxidative muscle metabolism than the lines CON and LDR.
In addition, characteristics of M. rectus femoris did not differ with the exception of a lower muscle mass in HBW offspring at age 180 d when born to mothers fed a HLeu diet during pregnancy. We did not observe differences in biochemical muscle characteristics with either pre- or postnatal exposure until weaning to maternal HLeu diet.

Energy expenditure components
We measured components of energy expenditure in C-C, C-HP and HP-C offspring of all 3 lines 1-2 days after weaning (22 d), at 42 d, and at 160 and 370 d.
Reduced body mass in male C-HP offspring at age 22 d was observed in all 3 mouse lines. These differences were only reflected by differences in components of energy metabolism in the HBW line. HBW offspring exposed to maternal HP diet either in utero or during suckling showed lower fat oxidation and higher carbohydrate oxidation rates.
Offspring group C-HP at 42 d in CON and HBW lines showed the lowest body mass. Following the same treatment LDR offspring did not show a significant body mass change. No diet effect was observed on energy expenditure, RQ, fat oxidation and energy retention. The HBW oxidized less fat than the other two genotypes. Energy retention did not differ from zero energy balance.
Energy expenditure was lower in C-HLeu offspring in the HBW line at 22 and 42 d, as was the case in 22 d offspring of the LDR, and the 42 d offspring of the CON line, respectively, in spite of unchanged body mass at this age (Table 2). Correspondingly, energy retention was higher in HBW offspring from the C-HLeu group. This observation seems to be related to a higher growth rate of CON and HBW offspring starting at 42 days of age in HBW and 63 days in CON, and persisting until age 125 d in HBW and age 300 d in CON. However, no diet-related differences could be observed at age 160 and 370 d. Taken together, this seems to indicate that CON offspring, and to a lower degree also HBW offspring, exposed to a C dam in utero but suckled by dams fed HLeu diet throughout pregnancy and lactation grow more and longer beyond the phase of early rapid growth (from birth until age 63 days;

i.e. 70 to 85% of adult body weight). These results indicate that genotypes behave differently in regard to a high leucine diet exposure during suckling.
Whole body energy expenditure using indirect calorimetry was measured in a subset of male offspring exposed to HP diet during early life at ages 160 and 370 d. Evaluation of selected data indicate no differences in components of energy metabolism due to diet. However, it appears that there are line differences. Likewise, in all 3 lines exposed to maternal high leucine (HLeu) diet during pregnancy and or lactation (C-C, HLeu-C, C-HLeu) there were no differences in the measured components of energy expenditure at ages 160 and 370 days.

Specific main results are summarized below.

High-protein diet during gestation and lactation affects mammary gland mRNA abundance, milk composition and pre-weaning litter growth in mice
We evaluated the effect of a high-protein diet (HP) on pregnancy, lactational and rearing success in mice. At the time of mating, females were randomly assigned to isoenergetic diets with HP (40% w/w) or control protein levels (C; 20%). After parturition, half of the dams were fed the other diet throughout lactation resulting in four dietary groups: CC (C diet during gestation and lactation), CHP (C diet during gestation and HP diet during lactation), HPC (HP diet during gestation and C diet during lactation) and HPHP (HP diet during gestation and lactation). Maternal and offspring body mass was monitored. Measurements of maternal mammary gland (MG), kidney and abdominal fat pad masses, MG histology and MG mRNA abundance, as well as milk composition were taken at selected time points. HP diet decreased abdominal fat and increased kidney mass of lactating dams. Litter mass at birth was lower in HP than in C dams (14.8 v. 16.8 g). Dams fed an HP diet during lactation showed 5% less food intake (10.4 v. 10.9 g/day) and lower body and MG mass. On day 14 of lactation, the proportion of MG parenchyma was lower in dams fed an HP diet during gestation as compared to dams fed a C diet (64.8% v. 75.8%). Abundance of MG a-lactalbumin, b-casein, whey acidic protein, xanthine oxidoreductase mRNA at mid-lactation was decreased in all groups receiving an HP diet either during gestation and/or lactation. Milk lactose content was lower in dams fed an HP diet during lactation compared to dams fed a C diet (1.6% v. 2.0%). On days 14, 18 and 21 of lactation total litter mass was lower in litters of dams fed an HP diet during lactation, and the pups' relative kidney mass was greater than in litters suckled by dams receiving a C diet. These findings indicate that excess protein intake in reproducing mice has adverse effects on offspring early in their postnatal growth as a consequence of impaired lactational function.
Görs S, Kucia M, Langhammer M, Junghans P, Metges CC. Technical note: Milk composition in mice—Methodological aspects and effects of mouse strain and lactation day. J Dairy Sci. 2009, 92:632–637
Kucia M, Langhammer M, Görs S, Albrecht E, Hammon HM, Nürnberg G, Metges CC. High-protein diet during gestation and lactation affects mammary gland mRNA abundance, milk composition and pre-weaning litter growth in mice Animal. 2010, epub Doi:10.1017/S1751731110001734

Maternal liver proteome profile during pregnancy, lactation and weaning
How high dietary protein affects global metabolism to adjust food intake is incompletely understood, particularly under physiological challenging conditions such as lactation. In order to identify these molecular events, mice were fed a high-protein (HP) diet from pregnancy, during lactation until weaning and compared with control fed counterparts. Liver specimens were analyzed for regulated proteins using 2-DE and MALDI-TOF-MS and plasma samples for metabolites. Based on the 26 differentially expressed proteins associated with depleted liver glycogen content, elevated urea and citrulline plasma concentrations, we conclude that HP feeding during lactation leads to an activated amino acid, carbohydrate and fatty acid catabolism while it activates gluconeogenesis. From pregnancy to lactation, plasma arg, trp, ser, gln and cys decreased, whereas urea concentrations increased in both groups. Concomitantly, hepatic glycogen content decreased while total fat content remained unaltered in both groups. Consideration of 59 proteins differentially expressed between pregnancy and lactation highlights different strategies of HP and control fed mice to meet energy requirements for lactation by adjusting amino acid degradation, carbohydrate and fat metabolism,

citrate cycle, but also ATP-turnover, protein folding, secretion of proteins and (de)activation of transcription factors.
Kuhla B, Kucia M, Görs S, Albrecht D, Langhammer M, Kuhla S, Metges CC. Effect of a high-protein diet on food intake and liver metabolism during pregnancy, lactation and after weaning in mice. Proteomics. 2010 Jul;10(14):2573-88

Hepatic expression of the GH/JAK/STAT/IGF pathway, Acute Phase Response signalling and Complement System are affected in mouse offspring by pre- and early postnatal exposure to maternal high protein diet

Effects of pre- and early postnatal exposure to maternal high protein diets are not well understood. Transcription profiling was performed in male mouse offspring exposed to maternal high protein diet during pregnancy and/or lactation to identify affected hepatic molecular pathways. Dams were fed isoenergetic diets with control (20% w/w) or high protein levels (40%). The hepatic expression profiles were evaluated by differential microarray analysis three days (d3) and three weeks (d21) after birth. Animals from three different high protein dietary groups, HP (d3, high protein diet during pregnancy), HPHP (d21, high protein diet during pregnancy and lactation) and CHP (d21, control diet during pregnancy and high protein diet during lactation), were compared with respective controls. Results: Offspring body and liver masses of all high protein groups were decreased.
Prenatal exposure to high protein diet affected hepatic expression of genes mapping to the Acute Response/Complement System and the GH/JAK/STAT/IGF signalling pathways. Maternal exposure to high protein diet during lactation affected offspring hepatic gene expression of the same pathways but additionally affected numerous genes mapping to protein, fatty acid, hexose and pyruvate metabolism pathways. Conclusions: (1) Hepatic genes of the Acute Response/Complement System and GH/JAK/STAT/IGF signalling pathways were down regulated in offspring of dams exposed to high protein diets during pregnancy and/or lactation. (2) Genes related to nutrient and energy metabolism, however, were only affected when high protein diet was administered during lactation. (3) Modulation of the GH/JAK/STAT/IGF pathway might be responsible for growth retardation by maternal high protein diet.

Effects of a maternal high protein diet during pregnancy and lactation on offspring development, and muscle characteristics in mice up to 1 y

Body mass development, body fat accretion, food intake, blood metabolites, muscle characteristics and locomotive activity were studied in male offspring from birth to 58 weeks to explore changes induced by pre- or postnatal exposure to a maternal high protein diet. We further investigated whether prenatal or early postnatal exposure (suckling period) to a maternal high protein diet has a larger effect on offspring development in mice. Mouse dams were fed a high protein (HP, 40%) or a control protein (C, 20%) diet from mating to weaning (21d). After birth litters were standardized to 10 pups and were cross-fostered to different dams fed C or HP, and three groups of offspring that were exposed to different prenatal/pre-weaning diet combinations: C-C, C-HP, and HP-C. Pregnant dams fed a HP diet had a 25-40% lower body mass gain and gave birth to 13% lighter litters with one pup less (11 vs. 12) whereas individual birth mass was not always lower ($P < 0.05$). At weaning until age 175 days C-HP pups had decreased body mass which did not persist until 1 y of age. At weaning nose to anus length was lower, and relative kidney and heart mass was higher in C-HP offspring compared to the other two groups; differences disappeared thereafter. Total body fat % as determined by DEXA was irrespective of the maternal diet but body fat accretion was less in C-HP offspring between 29 and 370 dpn ($P<0.05$). Plasma triglyceride and cholesterol values numerically decreased with age in C-HP offspring in contrast to the other groups where no change or an increase was observed. At 23 d, offspring exposed to a maternal HP diet during suckling experienced a reduced muscle growth whereas possibly negative effects due to exposure to a HP diet in utero can be ameliorated by rearing at a C dam. The effect of early life HP maternal diet exposure on muscle in 180 d old mice was small. Locomotive activity decreased from 185 to 360 dpn with no diet effect. We conclude that a maternal HP diet has immediate negative effects on maternal body mass and rearing performance as well as on offspring early postnatal development when suckled by a dam fed HP diet during pregnancy and lactation. However, a maternal HP diet during pregnancy or lactation did not cause persistent adverse effects.