Formula feeding alters hepatic gene expression signature, iron and cholesterol homeostasis in the neonatal pig

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Ronis MJJ, Chen Y, Shankar K, Gomez-Acevedo H, Cleves MA, Badeaux J, Blackburn ML, Badger TM. Formula feeding alters hepatic gene expression signature, iron and cholesterol homeostasis in the neonatal pig. Physiol Genomics 43: 1281–1293, 2011. First published September 27, 2011; doi:10.1152/physiolgenomics.00055.2011.—In the U.S. formula feeding remains more popular than breast-feeding. In the current study, neonatal piglets were breast fed and compared with those fed commercially available milk-based formula (milk) or soy-based formula (soy) from postnatal day 2 (PND2) until death at PND21 (the usual age of weaning). Liver weights were greater in formula-fed piglets (P < 0.05) than in breast-fed piglets (P > 0.05). Affymetrix array analysis revealed significant differences in hepatic gene expression profiles between piglets fed breast milk or formula, as well as between piglets fed milk or soy. In males, expression of 346 hepatic genes differed between formula-fed and breast-fed piglets, and soy-fed differed from milk-fed piglets in 277 genes. Furthermore, gene expression profiles of males differed from females, even when the same diet was consumed. Serum cholesterol was lower in piglets fed formula relative to breast-fed piglets (P < 0.05), and this was associated with elevations in mRNA encoding cholesterol 7a-hydroxylase (CYP7A1). Consistent with the human literature, breast-fed piglets had lower hepatic iron accumulation than formula-fed piglets. Hepcidin, a major regulator of hepatic iron trafficking, was elevated in piglets fed formula relative to breast-fed piglets (P < 0.05). Female piglets fed soy formula had increased expression of CYP3A enzymes (P < 0.05), and soy formula feeding decreased expression of several hepatic genes considered estrogen inducible. These data suggest that: 1) gene expression profiles in neonates differ significantly depending on the diet consumed, 2) hepatic iron storage and cholesterol metabolism clearly differ between breast and formula feeding in piglets, 3) there is no evidence that soy is estrogenic in neonatal pig liver.

Despite the AAP recommendations and initiation of breast-feeding in 62% of women, only 17% of babies in the U.S. are actually exclusively breast fed for 6 mo, and the majority of babies remain almost exclusively formula fed (47). Soy formulas currently occupy 15–20% of the U.S. formula market (6). Despite the large proportion of formula-fed babies and the enormous differences in dietary composition between breast milk and the different infant formulas, little research has examined the metabolic consequences of formula feeding.

One well-known metabolic difference between breast-fed and formula-fed infants relates to iron status. Breast milk is very low in iron relative to formulas (19), and iron deficiency has been documented in breast-fed infants in a number of different countries and in a number of recent randomized controlled trials after the first few months of life (11, 19, 21). Iron-fortified foods such as infant cereals are introduced during the second 6 mo of life to meet iron requirements in infants who continue to be fed breast milk (1). In addition to iron homeostasis, there are several studies that suggest that there are differences in lipid metabolism between breast-fed and formula-fed infants. This may relate to substantial differences in lipid composition between breast milk and formula. Breast milk is high in cholesterol and long chain saturated fats, while formula contains no cholesterol and substantial amounts of short chain saturated fats. Higher total and LDL-cholesterol values have been reported in breast-fed compared with cow’s milk-fed infants (5, 23). Moreover, increased urinary and fecal bile acid excretion has been reported in 3 mo old infants fed formula compared with those who were breast fed (30, 57).

There have been even fewer comparisons of metabolism between neonates fed cow’s milk- or soy-based formulas. This is despite data from experimental animal and clinical studies suggesting that soy consumption in early development has significant beneficial effects on body fat, lipid, and glucose homeostasis (Andres A, Gilchrist JM, Pivik RT, Casey PH, Badger TM, unpublished observations; 12). Soy formula feeding has also been reported to be accompanied by improvement in bone growth and composition in both infants and in a neonatal piglet model relative to breast-feeding (15, 57). Many investigators have attributed positive effects of soy on body composition and metabolism to estrogenic actions of isoflavones (12, 35, 36, 43, 46, 59).

Safety concerns regarding the use of soy formulas has focused on soy isoflavones because of their potential estrogenic actions during the neonatal period, including potential reproductive toxicity and increased incidence of hormonally sensitive cancers (e.g., breast, uterine, and endometrial cancers) (4, 14, 33, 53, 61). Such concerns have led to restrictions on the usage of soy formulas in countries such as France, Israel, and

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the United Kingdom and proposed new clinical studies by the National Institute for Environmental Health Sciences to investigate reproductive development in children fed soy formulas (4, 53). Although it is clear that pure isoflavones can have estrogenic actions in vitro and in vivo, there is little positive evidence that consumption of soy protein isolate (SPI), which is a complex mixture of proteins and >100 phytochemicals in addition to isoflavones, results in estrogenic actions in vivo in intact animals having endogenous circulating sex steroids (53). Moreover, there is a paucity of studies examining the potential estrogenicity of soy infant formula feeding in human infants (14, 26).

Soy feeding has been shown to have hypocholesterolemic effects in many animal models and in clinical studies in adults (4, 38, 51). The molecular mechanisms underlying this effect appear to involve both reduced cholesterol absorption from the gut and increased conversion to bile acids and transport into bile. This may involve effects on cholesterol metabolism and transport mediated through the nuclear receptors LXR, FXR, and PXR (51). Much less is known regarding the effects of soy formula consumption on cholesterol homeostasis in infants, although there is one report that urinary bile acid excretion is increased in soy formula-fed compared with breast-fed infants (58).

The current study was designed to examine the physiological, biochemical, and metabolic effects of cow’s milk and soy formula feeding compared with breast-feeding in a neonatal piglet model and to attempt to relate these effects to changes in hepatic gene expression using a genomics, array-based approach.

MATERIALS AND METHODS

Animal experiments. In the experiment previously published by Chen et al. (15), large White × Dutch Landrace × Duroc sows were fed grain-based, soy-free pig chow and were artificially inseminated. Parturition was induced on gestation day 111 by intramuscular (IM) lutealyse. Piglets from four litters were given an IM injection of iron-dextrane at birth and were allowed to suckle for 48 h before being randomly distributed between three groups of approximately equal mean weight. Five male and five female breast-fed piglets (Sow) were placed with sows for the duration of the experiment and allowed to feed ad libitum. Five male and five female piglets were fed cow’s milk-based formula (Milk) (Similac Advance powder;Ross Products, Abbott Laboratories, Columbus, OH). Six male and four female piglets were fed soy-based formula (Soy) (Enfamil Prosobee Lipil powder; Mead Johnson Nutritional, Evansville, IN). Formula diets were modified to meet the energy and nutrient recommendations of the National Research Council (NRC) for growing pigs (31), and diet composition was identical to that previously published (15). Formulated piglets were grouped housed by sex and were trained to drink from small bowls on a fixed schedule as described previously, to provide 1.047 MJ/kg/day until death on postnatal day (PND) 21 (31). All animals were housed in the animal facilities of the Arkansas Children’s Hospital Research Institute, an Association for the Assessment and Accreditation of Laboratory Animal Care-approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences (Little Rock, AR). Pigs were killed at 0800–1000 h, 6–8 h after the final feeding period, by exsanguination after anesthesia with isoflurane. Blood was collected and serum prepared by centrifugation. In addition, liver samples were formalin-fixed or were snap-frozen in liquid nitrogen. Frozen livers were stored at −70°C until use.

Serum biochemistry. Serum glucose was measured with Glucose Reagent (IR071-072; Synermed, Westfield, IN). Serum insulin was determined using an ELISA kit from Linco Research (St. Charles, MO). Serum triglycerides and total cholesterol were measured using commercially available reagents (IR140-R and IR060-R, Synermed). Nonesterified free fatty acids (NEFA) were measured using the NEFA C kit from Waco Chemicals (Richmond, VA).

Liver biochemistry. Hepatic protein content was determined using the BCA kit (Pierce, Rockford, IL). Hepatic DNA was extracted with Trizole reagent and quantitated by measuring UV absorbance at 260 nM. Hepatic glycogen stores were measured using Perlin’s Prussian blue stain and quantified by image analysis (9). Liver microsomes were prepared by differential ultracentrifugation using the method of Chipman and Walker (16). CYP3A-dependent microsomal monoxygenase activity was determined using three different prototypic substrates: erythromycin N-demethylase and testosterone 6β hydroxylase were measured as described by Romis et al. (48) and by use of the luciferin-IPA CYP3A4 P450-GLO assay (Promega) as described by the manufacturers. CYP3A apoprotein expression was quantified in liver microsomal preparations by Western immunoblot using a rabbit polyclonal antibody directed against rat CYP3A1 (20), the gift of Dr. Magnus Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden), and confirmed using mouse monoclonal antibodies directed against human CYP3A4/5 (Mab A254, Gentest). Phospho-STAT3, total STAT3 protein, phospho-Smad 1/5/8, and Smad 4 protein were quantified in Western blots of whole liver homogenates using antibodies from Cell Signaling (Danvers, MA) as described by Gao et al. (24) and Chen et al. (15).

Microarray analysis. Microarray preparation and data analysis were performed following Minimum Information About Microarray Experiments (MIAME)-supportive relational database (8). Total hepatic RNA was isolated using TRI reagent (Molecular Research Center) and purified using RNeasy mini columns as described previously (55). For each diet group and sex equal amounts of DNase-treated RNA from separate animals were combined into three pools as follows: for groups of n = 4 (pools contained 1, 1, and 2 individual animals) for groups of n = 5 (pools contained 1, 2, and 2 individual animals) and for groups of n = 6 (pools contained 2 individual animals each). First- and second-strand cDNA synthesis, biotin-labeled cRNA synthesis, fragmentation of cRNA and hybridization reactions were performed using one-cycle cDNA synthesis kit (Affymetrix). Briefly, 8 μg of purified RNA was used to synthesize cRNA. Labeled cRNA was synthesized from cDNA using a GeneChip IVT labeling kit (Affymetrix) according to the manufacturer’s instructions. cRNA (20 μg) was then fragmented in 5× fragmentation buffer for 35 min at 94°C. cRNAs were hybridized to an individual Affymetrix GeneChip Porcine arrays (n = 3/treatment) for 16 h at 45°C in the hybridization oven set at 60 rpm. The probe array was washed and stained using GeneChip fluids station 450 and scanned using GeneChip Scanner 3000.

Microarray validation by real-time RT-PCR. Total RNA (1 μg) was reverse-transcribed using iSCRIPT cDNA synthesis kit (Bio-Rad) following the manufacturer’s instructions. cDNA samples were amplified using previously described conditions (55). Expression of 18S and several housekeeping genes, including GAPDH, RP513, and cyclophilin, was examined between groups (18). RPS13 mRNA was found to be the least variable housekeeping gene across sexes and treatments in the neonatal pig liver and was utilized to normalize all real-time RT-PCR data. Primer sequences for genes quantified by real-time RT-PCR and further compared between treatment groups are shown in Table 1. Using values from a total of 45 genes quantified by real-time RT-PCR, we developed a model to investigate the relationship between fold-changes in the microarray and real-time RT-PCR data using a generalized linear model. The final model was
log \( Y = 0.905 \log X + 0.09 \), where \( X \) denotes the fold-change measured by Affymetrix microarrays and \( Y \) denotes the fold-change of the corresponding genes measured by real-time RT-PCR. This model has an Akaike’s information criterion (AIC) value of 110, which is smaller than the AIC value from normal linear regression (AIC = 343). The slope coefficient suggests that a unit increase in the log-fold change obtained by real-time RT-PCR for each of the selected genes measured by Affymetrix microarrays and \( Y \) denotes the fold-change was associated with 0.905-fold increase in the log-fold change obtained by real-time RT-PCR for each of the selected genes. Since the slope in this model is almost equal to 1, we concluded that the microarray data provide a relatively accurate reflection of overall patterns of gene expression in neonatal pig liver.

**Statistical analysis.** The data files (.CEL files) containing the probe level intensities from the Affymetrix arrays were processed using the Robust Multiarray Analysis algorithm (55) for background correction, normalization, and log2 transformation using GeneSpring 7.3 (Agilent Technologies). Subsequently, the data were subjected to per-chip and per-gene normalization using GeneSpring. Within each sex comparisons were made between treatments: formula vs. sow-fed and milk- vs. soy-fed groups, separately. Genes were filtered based on minimum \( \pm 1.5 \)-fold change and \( P \) value \( \leq 0.05 \) using a paired Student’s \( t \)-test. Corrections for multiple testing were performed using the false discovery rate method (55). This list of differentially expressed genes was used to evaluate the pattern of gene expression profile by correlation-based hierarchical clustering. Known biological functions of genes were queried using Affymetrix NetAffx and Gene Ontology (GO) analyses for biological/molecular function performed using DAVID (34). For each outcome, a two-factor analysis of variance (ANOVA) with corrections for multiple testing was performed using the Stata statistical package version 12.0 (Stata, College Station, TX).

**Table 1. Primer sequences utilized in the current study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tbody>
<tr>
<td>TFRC-F</td>
<td>TGTCGTCGTCCTTGCTTGTCCTTG</td>
<td>CGACGCTCGACGCTCGACGCTCG</td>
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</table>
| TFRC-R    | TGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
three diet groups and the two sexes. Post hoc comparisons of diets, stratified by sex, were performed using all pairs Tukey-Kramer comparison test and were considered significant if \( P < 0.05 \). The generalized linear model was estimated with the glm procedure in R v. 2.12 (http://www.R-project.org).

RESULTS

Body weight gain and organogenesis. Body weight and weight gain were within normal limits and similar between all three diet groups but were significantly lower in females than males (Table 2). Two-way ANOVA indicated significant diet and sex effects on liver and kidney weight (Table 2). However, the effect of sex was related to the higher body weight of male piglets and disappeared when the data were normalized to body weight. Normalized liver weights were higher in both formula-fed groups compared with the sow groups of either sex (\( P = 0.0001 \)). The increase in liver weight after formula feeding was not accompanied by consistent increases in either DNA or protein content/g liver, suggesting that this effect is due to neither liver hypertrophy nor hyperplasia (Table 2). No increase was observed in mean liver glycogen content. In the male, normalized kidney weight of the Milk group was greater than either Sow or Soy groups (\( P < 0.05 \)). However, no significant differences were observed in kidney weight in the females (Table 2).

Glucose and lipid homeostasis. Serum glucose concentrations were unchanged across all groups in both sexes (Fig. 1A). Similarly, there were no significant differences in serum insulin levels between feeding groups in either sex (data not shown). Serum triglyceride concentrations were affected by

Table 2. Effects of formula feeding on piglet neonatal growth, liver, and kidney

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Wt., kg</th>
<th>Growth Rate, kg/day</th>
<th>Liver Wt., g</th>
<th>Liver Wt., % body wt.</th>
<th>Liver Protein, mg/g</th>
<th>Liver DNA, mg/g</th>
<th>Liver Glycogen, mg/g</th>
<th>Kidney Wt., g</th>
<th>Kidney Wt., % body wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sow</td>
<td>7.40 ± 0.30</td>
<td>0.30 ± 0.02</td>
<td>177 ± 8.7(a)</td>
<td>2.4 ± 0.10(a)</td>
<td>99 ± 12.6(a)</td>
<td>2.91 ± 0.10</td>
<td>46 ± 6.0</td>
<td>43 ± 2.1(c)</td>
<td>0.58 ± 0.06(c)</td>
</tr>
<tr>
<td>Milk</td>
<td>7.20 ± 0.40</td>
<td>0.30 ± 0.02</td>
<td>247 ± 9.0(a)</td>
<td>3.4 ± 0.10(a)</td>
<td>151 ± 11.9(b)</td>
<td>2.50 ± 0.50</td>
<td>43 ± 6.5</td>
<td>52 ± 2.5(c)</td>
<td>0.72 ± 0.03(c)</td>
</tr>
<tr>
<td>Soy</td>
<td>6.90 ± 0.50</td>
<td>0.20 ± 0.17</td>
<td>235 ± 9.8(a)</td>
<td>3.4 ± 0.10(a)</td>
<td>119 ± 12.6(b)</td>
<td>2.76 ± 0.70</td>
<td>57 ± 11.4</td>
<td>44 ± 6.2(b)</td>
<td>0.63 ± 0.02(b)</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sow</td>
<td>5.80 ± 0.30</td>
<td>0.20 ± 0.02</td>
<td>142 ± 9.0(a)</td>
<td>2.5 ± 0.1(a)</td>
<td>133 ± 11.2</td>
<td>3.24 ± 0.30</td>
<td>43 ± 13.0</td>
<td>36 ± 1.7</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>Milk</td>
<td>5.60 ± 0.40</td>
<td>0.20 ± 0.02</td>
<td>176 ± 9.9(b)</td>
<td>3.2 ± 0.1(b)</td>
<td>107 ± 15.2</td>
<td>2.95 ± 0.40</td>
<td>30 ± 3.8</td>
<td>38 ± 2.2</td>
<td>0.70 ± 0.06</td>
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<tr>
<td>Soy</td>
<td>6.50 ± 0.30</td>
<td>0.20 ± 0.02</td>
<td>237 ± 17.8(b)</td>
<td>3.7 ± 0.2(a)</td>
<td>89 ± 10.7</td>
<td>2.31 ± 0.30</td>
<td>43 ± 17.6</td>
<td>43 ± 2.9</td>
<td>0.67 ± 0.08</td>
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\( P \) Values

<table>
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<tr>
<th>Diet</th>
<th>0.71</th>
<th>0.38</th>
<th>0.0001</th>
<th>0.0001</th>
<th>0.17</th>
<th>0.51</th>
<th>0.22</th>
<th>0.036</th>
<th>0.005</th>
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<tr>
<td>Sex</td>
<td>0.0001</td>
<td>0.01</td>
<td>0.0005</td>
<td>0.85</td>
<td>0.21</td>
<td>0.77</td>
<td>0.13</td>
<td>0.001</td>
<td>0.049</td>
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<tr>
<td>Inter</td>
<td>0.32</td>
<td>0.05</td>
<td>0.009</td>
<td>0.1</td>
<td>0.01</td>
<td>0.59</td>
<td>0.72</td>
<td>0.60</td>
<td>0.68</td>
</tr>
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</table>

Data are means ± SE. Means with different superscripts were significantly different between diets, \( P < 0.05 \), \( a < b < c \).

Fig. 1. Glucose and lipid homeostasis in breast-fed and formula-fed piglets on postnatal day (PND) 21. A: serum glucose concentration; B: serum triglyceride concentration; C: serum nonesterified free fatty acid (NEFA) concentration; D: serum total cholesterol concentration. Data are means ± SE. Means with differing subscripts are significantly different for diet within sex (\( P < 0.05 \), \( a < b \).
diet ($P = 0.009$) and were reduced in the Milk group compared with the Soy group particularly in male piglets ($P < 0.05$) (Fig. 1B). Serum NEFA levels were also diet dependent ($P = 0.003$) and were lower in the Milk group compared with the Sow group, particularly in males (Fig. 1C). In contrast, total cholesterol values were lower in both formula-fed groups relative to the Sow group ($P < 0.001$), particularly in female piglets (Fig. 1D).

**Hepatic iron storage.** Measurement of hepatic iron storage by Perl’s Prussian blue staining of fixed liver sections revealed robust staining in both formula-fed groups in both sexes but no detectable hepatic iron in the Sow groups ($P < 0.0001$) (Figs. 2 and 3).

**Hepatic gene expression profiles in formula-fed vs. sow-fed piglets.** To determine if the physiological and biochemical changes observed in formula-fed piglets could be explained on the basis of change in hepatic gene expression and to probe differences between the feeding groups in more molecular detail, we ran microarray analysis using Affymetrix Porcine arrays. The microarray data are available as accession number GSE28349 in the Gene Expression Omnibus repository at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/). Hierarchical clustering revealed significant sex-differences in hepatic gene expression in Sow- and formula-fed piglets even at this early age prior to puberty or sexual dimorphism in endocrine profiles (Fig. 4). A comparison was made between expression data in the Sow groups ($n = 3/sex$) and expression data from all piglets fed formula irrespective of formula type ($n = 6/sex$) (Fig. 4). Relative to expression in the Sow group, 346 genes were significantly changed >1.5-fold by formula feeding in male piglets and 279 genes were significantly changed >1.5-fold by formula feeding in female piglets (Fig. 4A). A subset of 98 genes significantly changed >1.5-fold in the same directions in formula-fed compared with Sow groups independent of sex. This group of genes, 71 upregulated and 27 downregulated, represents the most robust difference in gene expression between sow and formula feeding. Functional annotation clustering was conducted using DAVID. Eight common gene clusters were found in both sexes and included genes encoding metal cation binding proteins, many involved in redox reactions; genes encoding proteins involved in fatty acid metabolism; endoplasmic reticulum membrane proteins; proteins involved in inflammatory processes; endopeptidase inhibitors; signal peptides; and nucleotide binding and transmembrane proteins. Five gene clusters were identified as male specific, and six clusters were identified as being female specific. These included genes for cation binding proteins, membrane proteins, and signal peptides, but male-specific genes also included nucleotide binding proteins and genes associated with apoptosis, while female-specific genes included phosphoproteins and proteins associated with the Golgi apparatus.
The most highly upregulated of the 96 genes common to formula feeding in both sexes are shown in Fig. 4C. We have verified many of the hepatic gene changes associated with formula feeding relative to breast-feeding using real-time RT-PCR. The most highly upregulated and downregulated genes were HAMP (hepcidin) (increased 80–150-fold) \( (P = 0.003) \), more in females than males \( (P = 0.02, \text{sex difference}) \), and TFRC (the transferrin receptor) (decreased 70–80%) in both formula-fed groups, in both sexes \( (P = 0.0001) \) (Table 3). These two proteins are known to be reciprocally regulated and orchestrate systemic iron fluxes controlling hepatic iron storage and plasma iron levels (32). In adults, hepcidin expression has been suggested to be induced through two signaling pathways: BMP/Smad signaling mediating a feedback loop sensing systemic iron availability and JAK/STAT3 signaling mediating the effects of inflammation (30). Therefore, we measured ratios of p-STAT3/total STAT3 and p-Smad(1, 5, 8)/Smad4 ratios in liver homogenates by Western blot analysis (Table 3). No significant effects of formula feeding were observed on pSTAT3/STAT3 ratio. However, p-Smad/Smad4 ratio was affected by diet \( (P = 0.006) \) and was increased in the Soy group compared with other diet groups and increased in the Milk relative to the Sow group \( (P < 0.05) \) in male piglets. An additional cluster of genes regulating lipid metabolism were confirmed to be upregulated in both formula-fed groups relative to the Sow group (Table 3). Stearoyl CoA desaturase (SCD) was increased three- to sevenfold by formula feeding \( (P = 0.001) \) and catalyzes the conversion of saturated to monounsaturated fatty acids, an important step in conversion of fatty acids into triglycerides (7). Other significantly upregulated enzymes in liver of formula-fed piglets of both sex included 3-hydroxy-3-methylglutaryl-Co-A synthase and reductase (HMGCS1 and HMGR), isopentylidiphosphate delta isomerase (IDi1), and lanosterol \( 14/\text{H}9251 \) demethylase (CYP51), which are all part of the mevalonate pathway of endogenous cholesterol synthesis and cholesterol \( 7/\text{H}9251 \) hydroxylase (CYP7A1), the rate-limiting step in the conversion of cholesterol to bile acids.

An additional comparison was made between the two formula-fed groups of piglets to determine formula-specific changes in neonatal hepatic gene expression. In male piglets, 257 genes were changed >1.5-fold by Soy. In female piglets, Soy changed expression of a 267 genes by >1.5-fold (Fig. 5, A and B). Interestingly, overlap between the two sets of genes representing sex-independent effects of soy was restricted to a subset of only 34 genes that changed in the same direction in both sexes, 15 upregulated and 19 downregulated (Fig. 5C). Functional annotation clustering using DAVID revealed only
one common cluster: six metal cation binding proteins. Eight gene clusters were identified as male specific, and seven clusters as female specific. The largest male-specific and female-specific clusters also included metal cation binding genes, but these appeared to have different functions. The male-specific cluster included genes encoding calcium binding proteins, while the female cluster included genes encoding iron binding proteins and those involved in oxidation/reduction reactions such as cytochrome P450 enzymes. Other large clusters identified as both male specific and female specific

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Hepcidin</td>
<td>1.0 + 0.6</td>
<td>139.4 + 32.7</td>
</tr>
<tr>
<td>TFRG</td>
<td>1.0 + 0.2</td>
<td>1.1 + 0.2</td>
</tr>
<tr>
<td>P-STAT3/STAT3</td>
<td>1.0 + 0.1a</td>
<td>6.3 + 2.1b</td>
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</tbody>
</table>

Data are means ± SE. Means with differing superscripts are significantly different (P < 0.05); a < b < c. 1mRNA expression/housekeeping gene RPS13 expressed relative to expression in sow-fed male = 1.0 as determined by real-time RT-PCR. TFRG, transferrin receptor; SCD, stearoyl CoA desaturase; IDI1, isopentyldiphosphate delta isomerase; CYP51, lanosterol 14a demethylase; HMG1, HMG reductase; HMGCS1, HMG CoA synthase; CYP7A1, cholesterol 7a-hydroxylase. 2Western blot analysis of phosphorylated STAT3/total STAT3 protein and phosphorylated Smad (1,5,8)/total Smad 4 protein.

Fig. 5. Differential hepatic gene expression signatures in milk formula- and soy formula-fed piglets on PND21. A: Venn diagram of differentially expressed genes in male and female piglets. B: hierarchical cluster analysis of all hepatic genes in male and female milk formula-fed compared with soy formula-fed piglets. Colors: red>orange represent upregulation; yellow represents no relative effect; blue>green represent downregulation. C: hierarchical cluster analysis of hepatic genes altered (≥ 1.5-fold, P < 0.05) in both male and female soy formula-fed compared with milk formula-fed piglets.
Table 4. Specific effects of soy formula relative to cow’s milk formula on gene expression in neonatal piglets of both sexes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Milk Male</th>
<th>Soy Male</th>
<th>Milk Female</th>
<th>Soy Female</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGD</td>
<td>1.00 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.50 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.21 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.62 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ARG1</td>
<td>1.00 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.21 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.85 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.95 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TXNIP1</td>
<td>1.00 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.005</td>
</tr>
<tr>
<td>A2M1</td>
<td>1.00 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MT3</td>
<td>1.00 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.02 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data are means ± SE. Means with differing superscripts are significantly different between diets. <sup>a</sup> and <sup>b</sup> represent relative expression in sow-fed male and soy-fed male, respectively. 

Anti-rat CYP3A1 antibody. CYP3A apoprotein expression was elevated in Soy females relative to the other two diet groups only in female piglets <sup>P < 0.05</sup>. CYP3A protein expression patterns were conformed with a mouse monoclonal antibody to human CYP3A4/5 (data not shown). We verified that the changes in CYP3A gene and apoprotein expression were biologically meaningful by measuring CYP3A-dependent mono-oxygenase activities in the pig liver microsomes using three different substrates: erythromycin N-demethylase, testosterone 6b-hydroxylase, and the luciferin-IPA CYP3A4 P450-GLO assay (Fig. 7, B–D). All three activities were significantly affected by diet <sup>P < 0.05</sup>. Erythromycin N-demethylase mirrored the pattern of CYP3A apoprotein expression most closely and was induced in Soy piglets relative to Sow and Sox.

Fig. 6. Effects of breast and formula feeding on expression of hepatic CYP3A mRNA species in piglets on PND21. A: male, B: female. Data represent ratios of target mRNA/RPS13 housekeeping gene relative to sow-fed piglets of each sex = 1.0, as determined by real-time RT-PCR. Data are means ± SE. Means with differing subscripts are significantly different <sup>P < 0.05</sup> for diet within sex, a < b.
Milk groups only in female piglets (P < 0.05) (Fig. 7C). Testosterone 6β-hydroxylase was also induced relative to the Milk group only in females (P < 0.05) (Fig. 7D).

Hepatic gene expression profiles of known estrogen and peroxisome proliferator-activated receptor-α-regulated genes in formula vs. sow groups. It has been suggested that soy products and soy-associated isoflavones are capable of activating estrogen-regulated and peroxisome proliferator-activated receptor (PPAR)-regulated pathways (12, 33, 45, 51). To assess if these pathways were affected by soy formula feeding in the neonatal pig, we conducted real-time RT-PCR analysis to examine expression of six genes involved in glucose and lipid metabolism that have been previously shown to be highly estrogen responsive in rodent liver: Ptdgs, pyruvate dehydrogenase kinase isozyme 4 (PDK4), Pck1 (PEPCK), STAT3, fatty acid synthase (FASN), and glucose-6-phosphatase (G6Pc) (24, 25, 40). In addition, we examined two genes previously shown to be responsive to both estrogens and PPARα itself and acyl Co-A oxidase (ACOX) (25, 51) and three other PPARα-regulated genes [carnitine palmitoyltransferase 1 (CPT-1), CYP4A21, and CYP4A24] (39, 51) (Table 5). In mouse liver, PTGDS, PDK4, Pck1, and STAT3 have all been reported to possess estrogen-response elements and to be upregulated via estrogen responsive in rodent liver. Ptdgs, pyruvate dehydrogenase kinase isozyme 4 (PDK4), Pck1 (PEPCK), STAT3, fatty acid synthase (FASN), and glucose-6-phosphatase (G6Pc) (24, 25, 40). In addition, we examined two genes previously shown to be responsive to both estrogens and PPARα itself and acyl Co-A oxidase (ACOX) (25, 51) and three other PPARα-regulated genes [carnitine palmitoyltransferase 1 (CPT-1), CYP4A21, and CYP4A24] (39, 51) (Table 5). In mouse liver, PTGDS, PDK4, Pck1, and STAT3 have all been reported to possess estrogen-response elements and to be upregulated via estrogen responsive in rodent liver. Ptdgs, pyruvate dehydrogenase kinase isozyme 4 (PDK4), Pck1 (PEPCK), STAT3, fatty acid synthase (FASN), and glucose-6-phosphatase (G6Pc) (24, 25, 40). In addition, we examined two genes previously shown to be responsive to both estrogens and PPARα itself and acyl Co-A oxidase (ACOX) (25, 51) and three other PPARα-regulated genes [carnitine palmitoyltransferase 1 (CPT-1), CYP4A21, and CYP4A24] (39, 51) (Table 5). In mouse liver, PTGDS, PDK4, Pck1, and STAT3 have all been reported to possess estrogen-response elements and to be upregulated via estrogen responsive in rodent liver. Ptdgs, pyruvate dehydrogenase kinase isozyme 4 (PDK4), Pck1 (PEPCK), STAT3, fatty acid synthase (FASN), and glucose-6-phosphatase (G6Pc) (24, 25, 40). In addition, we examined two genes previously shown to be responsive to both estrogens and PPARα itself and acyl Co-A oxidase (ACOX) (25, 51) and three other PPARα-regulated genes [carnitine palmitoyltransferase 1 (CPT-1), CYP4A21, and CYP4A24] (39, 51) (Table 5). In mouse liver, PTGDS, PDK4, Pck1, and STAT3 have all been reported to possess estrogen-response elements and to be upregulated via estrogen responsive in rodent liver.

In the current study in neonatal pig liver, these genes were either unaffected by soy formula feeding relative to cow’s milk formula or breast-feeding or expression was significantly suppressed. FSN and G6Pc mRNAs have both been reported to be repressed by estrogen treatment, with FSN downstream of activated STAT3 signaling (24, 25, 40). In neonatal pig liver, FASN mRNA expression was increased in the Soy group relative to the Milk group, particularly in the male (P < 0.05), and expression of G6Pc mRNA was not significantly changed (Table 5). The CYP4A21 gene, suggested to be regulated by PPARα was increased in the Soy relative to Milk or Sow groups in both sexes (P < 0.05) (Table 5). However, no consistent soy effects were observed on expression of other PPARα-responsive genes such as the transcription factor itself, ACOX, CPT-1, and CYP4A24.

**DISCUSSION**

The neonatal piglet is a good animal model for the human infant, with similar growth, development, metabolism, and endocrine systems. Unlike rodents, piglets may also be breast fed or fed human infant formulas almost immediately after birth. This allows the use of neonatal piglets to model the physiological, biochemical, and molecular effects of human formula feeding (15, 31). In addition, unlike rodents or monkeys, but just like human infants, neonatal piglets do not convert the soy isoflavone daidzein into the potent estrogen equol (28). Therefore, the neonatal piglet is the most appropriate model to study the specific effects of soy infant formula relative to cow’s milk formula. In the current study, we utilized this model to examine the effects of formula feeding on metabolism and hepatic gene expression.

Although recent reports have suggested improved glucose tolerance in mice fed soy postnatally (12), serum glucose and insulin values were unchanged in breast-fed vs. formula-fed piglets at death on PND21. However, lipid homeostasis was significantly different in formula-fed compared with breast-fed piglets. In general, serum NEFA values were lower after formula-feeding compared with breast-feeding. In adults, the majority of NEFA are generated from breakdown of triglycerides in adipose tissue by lipoprotein lipase (22) with a small additional component derived from dietary lipids through

![Fig. 7. Quantification of CYP3A apoprotein expression and CYP3A-dependent monooxygenase activities in sow-fed, milk formula-fed, and soy formula-fed piglets on PND21. A: immunoquantitation of CYP3A apoprotein expression in liver microsomes based on densitometric analysis of Western blots. B: luciferin-IPA CYP3A4-GLO assay. C: erythromycin N-demethylase. D: testosterone 6β hydroxylase. Data are means ± SE. Means with differing subscripts are significantly different (P < 0.05) for diet within sex, a < b.](http://physiolgenomics.physiology.org/content/43/22/1289/sci-artifacts/10.220.33.3)
lipase action on chylomicrons in the hepatic vascular bed (41). However, in the neonate, there is very little adipose tissue and the dietary component may be much more important in determining serum NEFA concentrations. In this regard, both formulas had 49% of total calories as fat compared with the reported 60% in sow milk and 29% of the formula fat was in the form of short chain fatty acids (C8–C12) from coconut oil, which undergo substantially different transport and metabolism compared with longer chain fatty acids (15). Serum triglyceride concentrations were found to be significantly higher in the Soy groups of both sexes compared with the Milk groups. It is possible that this reflects increased endogenous fatty acid synthesis in the liver since hepatic FASN mRNA expression was also found to be higher in both Soy groups compared with Sow groups.

Serum total cholesterol concentrations were significantly lower in both formula-fed groups relative to breast-fed piglets. This finding is consistent with clinical reports (5, 23). Interestingly, the hepatic gene array data demonstrate significant upregulation of mRNAs encoding enzymes in the mevalonate pathway, important in endogenous cholesterol synthesis, in piglets fed either cow’s milk or soy formulas, relative to breast-feeding. This presumably reflects the lack of cholesterol in infant formulas and the requirement for cholesterol in important endogenous processes during early development such as an important component of cell membranes and in hormone synthesis. Increases in the mevalonate pathway were accompanied by even larger increases in expression of CYP7A1 mRNA. CYP7A1 catalyzes the rate-limiting step in the formation of bile acids from cholesterol. These data suggest that formula feeding may also stimulate bile acid synthesis from endogenous cholesterol. Interestingly, increased bile acid excretion has been observed in infants fed cow’s milk and soy formula compared with breast-feeding (30, 58) and in hamsters fed soy protein (38). These data are also consistent with the hypcholesterolemic effects associated with soy feeding in adults (4, 51). The increases in hepatic CYP7A1 mRNA expression in soy formula-fed piglets relative to both breast- and milk formula-fed animals is similar to that previously reported by our laboratory in weaning rats fed soy protein isolate compared with casein. In that study, we demonstrated that soy feeding resulted in activation of the nuclear liver X-receptor (LXR), which regulates cholesterol metabolism and transport (51). Interestingly, soy formula-specific increases in expression of hepatic CYP3A enzymes, which are also involved in bile acid metabolism, mirror previously reported induction of CYP3A in rodents fed soy protein isolate (48–50). This induction has recently been demonstrated to be mediated via increased binding of the pregnane X-receptor (PXR) another member of the nuclear receptor family to CYP3A promoters (52).

In agreement with a previous study in piglets, liver weight was significantly increased after formula feeding (31). However, it is unclear why this occurred, since neither protein, DNA, nor glycogen content/g liver changed significantly. Moreover, there were no differences in hepatic triglyceride content (data not shown). One possibility is that the degree of liver hydration differs perhaps as a result of different carbohydrate sources in sow milk and formulas. Significant differences in hepatic iron storage were detected between feeding groups using Perl’s Prussian blue staining and differences in liver size may also reflect this difference in iron status since dose-dependent increases in liver size with iron treatment have previously been reported in a mouse model of chronic iron overload (56). Both formula-fed groups of both sexes had greater hepatic iron staining, which was almost entirely absent in sow milk formula-fed animals is similar to that previously reported (15, 19). Hepatocytes are a major site of iron storage. This is orchestrated via the regulatory hormone hepcidin, which is also made in the liver and

**Table 5. Effects of formula feeding on expression of known E2- and PPAR-regulated genes involved in glucose and lipid metabolism in neonatal piglet liver**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Male Sow</th>
<th>Male Milk</th>
<th>Male Soy</th>
<th>Female Sow</th>
<th>Female Milk</th>
<th>Female Soy</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTGDS1</td>
<td>1.0 ± 0.2a</td>
<td>0.4 ± 0.1b</td>
<td>0.1 ± 0.1a</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.037</td>
</tr>
<tr>
<td>Pink1 (PEPCK)</td>
<td>1.0 ± 0.3a</td>
<td>2.2 ± 0.3b</td>
<td>0.9 ± 0.1a</td>
<td>2.2 ± 0.4</td>
<td>1.3 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>0.11</td>
</tr>
<tr>
<td>Pdk4</td>
<td>1.0 ± 0.2a</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.02</td>
<td>1.6 ± 0.6a</td>
<td>0.5 ± 0.2b</td>
<td>0.1 ± 0.02b</td>
<td>0.0005</td>
</tr>
<tr>
<td>STAT3</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.1a</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.2a</td>
<td>1.7 ± 0.2b</td>
<td>0.8 ± 0.1a</td>
<td>0.015</td>
</tr>
<tr>
<td>Fsn1</td>
<td>1.0 ± 0.2a</td>
<td>1.3 ± 0.1a</td>
<td>2.6 ± 0.5b</td>
<td>0.7 ± 0.1a</td>
<td>1.0 ± 0.2a</td>
<td>2.1 ± 0.1b</td>
<td>0.0001</td>
</tr>
<tr>
<td>G6pc</td>
<td>1.0 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**E2 Regulated**

<table>
<thead>
<tr>
<th>PPAR Inducible</th>
<th>Male Sow</th>
<th>Male Milk</th>
<th>Male Soy</th>
<th>Female Sow</th>
<th>Female Milk</th>
<th>Female Soy</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR alpha</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.2a</td>
<td>1.0 ± 0.1ab</td>
<td>0.8 ± 0.1b</td>
<td>0.22</td>
</tr>
<tr>
<td>Acox1</td>
<td>1.0 ± 0.1a</td>
<td>1.1 ± 0.1b</td>
<td>1.4 ± 0.1b</td>
<td>1.5 ± 1.0</td>
<td>1.3 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>0.028</td>
</tr>
</tbody>
</table>

**PPAR Inducible**

| Cpt-1          | 1.0 ± 0.2 | 1.3 ± 0.2 | 1.7 ± 0.3 | 0.7 ± 0.1a | 1.6 ± 0.3b | 1.5 ± 0.3ab | 0.02    |
| Cyp4a21        | 1.0 ± 0.2a | 2.4 ± 0.3b | 4.1 ± 0.5c | 0.7 ± 0.1a | 2.7 ± 0.3b | 6.3 ± 0.4c | <0.0001 |
| Cyp4a24        | 1.0 ± 0.2 | 0.9 ± 0.5 | 1.1 ± 0.2 | 0.5 ± 0.2 | 1.4 ± 0.6 | 1.4 ± 0.6 | 0.55    |

Data are means ± SE. Means with differing superscripts are significantly different between diets (P < 0.05), a < b < c. mRNA expression/housekeeping gene RPS13 expressed relative to expression in sow-fed male = 1.0 as determined by real-time RT-PCR. PTGDS, prostaglandin 2-synthase; PDK4, pyruvate dehydrogenase kinase isozyme 4; Pink1, phosphoenolpyruvate carboxykinase; STAT3, signal transducer and activator of transcription 3; FSN, fatty acid synthase; G6pc, glucose 6-phosphatase.
which promotes hepatic iron storage by binding to and degrading the iron exporter ferroportin. Another important protein in iron homeostasis is the transferrin receptor (TFRC), which supplies iron to cells by binding iron-loaded ferritin from the serum followed by endocytosis (32). Hepcidin was the most induced mRNA species and TFRC the most repressed mRNA in pig liver after formula feeding. This is consistent with the observed differences in hepatic iron storage between breast- and formula-fed piglets. There are two well-described cell signaling pathways whereby hepcidin is regulated: regulation via transferrin-Fe2+, which involves bone morphogenic protein (BMP) signaling through phosphorylation of the Smad proteins; and regulation via inflammation, which involves signaling through STAT3. Our data suggest that at least in the male piglets, hepatic Smad phosphorylation is increased by formula feeding compared with the Sow group (Table 3). This is consistent with previous data from our laboratory that demonstrated increased Smad phosphorylation in bone of formula-fed piglets (15). In contrast, there was no evidence for increased STAT3 signaling in liver of either cow’s milk or soy formula-fed piglets (Tables 3 and 5).

Some of the other gene expression differences observed in sow-fed and formula-fed piglet liver may also reflect differences in dietary macronutrient composition. For example, although protein and amino acid composition of the formula diets meet the NRC recommendations for the growing pig (31) and consisted of high-quality purified proteins, protein content of the formulas was still significantly lower than that found in sow milk (8–10% energy compared with 20% energy) (15). Surprisingly, gene array analysis of hepatic gene expression revealed significant sexual dimorphism in all three feeding groups. Although sex-dependent gene expression in liver is well known and has been thoroughly investigated in adults, it has generally been assumed that sexual dimorphism manifests itself at puberty and is associated with expression of sex steroid receptors, the rise in sex steroid production, and appearance of sexually dimorphic patterns of growth hormone secretion programmed by neonatal sex steroid surges (17). We have recently observed the same type of sexual dimorphic gene expression in the livers of prepubertal rats (54). In addition to sex differences in breast-fed piglets, gene array analysis suggested that there are sexually dimorphic responses to formula feeding. Only 98 of the 429 hepatic genes expressed in formula-fed piglets at levels >1.5-fold different from those in breast-fed piglets were sex independent. When milk and soy formula groups were compared, only 34 of the 466 genes significantly altered by soy formula feeding were sex independent. To some degree these sexually dimorphic responses simply reflect a matter of degree and are related to the chosen cut-off value of 1.5-fold. For example, significant induction of CYP7A1 and CYP3A29 mRNA expression was observed in both male and female piglets fed soy formula compared with cow’s milk. However, the level of induction was substantially greater in female piglets, and so both these genes appear in the female-specific gene list.

Although the hepatic response to soy formula feeding appears somewhat more sex-dependent than that to formula feeding overall, there is no evidence from the current data to suggest that this represents an estrogenic response associated with consumption of isoflavone phytoestrogens such as genistein and daidzein, which are found associated with soy protein. These data are consistent with previous data from our laboratory demonstrating dramatic differences in hepatic gene expression signatures in ovariectomized female rats in response to the feeding of SPI, the sole protein source in soy infant formula, relative to the response to 17β-estradiol restoration to physiologically relevant concentrations (55).

We and others have previously shown that consumption of SPI during early development results in a leaner phenotype in both rats and mice (3, 12, 51). Likewise, preliminary data from a longitudinal, prospective feeding study of breast-fed, cow’s milk- and soy formula-fed infants at the Arkansas Children’s Nutrition Center (The Beginnings Study), suggests that at 3 mo of age, all formula-fed infants grow within normal limits, but that soy-fed infants are leaner than cow’s milk-fed infants (Andres A, Gilchrist JM, Pivik RT, Casey PH, Badger TM, unpublished observations). It has been suggested that reduced adiposity results from estrogenic actions of isoflavones (12, 36, 43, 46). This would be consistent with recent reports that genetic rescue of nonclassical estrogen receptor signaling rescues estrogen receptor alpha−/− mice from obesity by increasing energy expenditure (44). Consistent with clinical studies in breast- and formula-fed infants (Andres A, Gilchrist JM, Pivik RT, Casey PH, Badger TM, unpublished observations), there were no differences in growth rate between the groups of piglets of the same sex fed different diets. Unfortunately, body composition was not measured in these animals, and so no conclusions can be drawn with regards to adiposity or energy expenditure from the current study.

In animal models, soy feeding in early development has also been shown to improve glucose homeostasis as a result of increasing systemic insulin sensitivity and to reduce hepatic triglyceride concentrations under conditions of obesity and high-fat feeding (3, 12, 51). Various mechanisms have been proposed for these effects including activation of signaling through PPAR pathways (45, 51), but estrogenic actions of soy isoflavones remain the favored explanation (12). Estrogen signaling in the liver increases expression and activation of signal transducer and activator of transcription (STAT)3, resulting in downstream inhibition of lipogenic genes such as fatty acid synthetase (FASN) and increased leptin signaling (24). Estradiol has also been shown to induce other hepatic genes involved in glucose and lipid homeostasis in mouse liver. These include prostaglandin D2 synthase (Ptgds), an enzyme responsible for synthesis of naturally occurring PPARγ ligands, and phosphoenolpyruvate carboxykinase (Pck1), the rate-limiting enzyme in hepatic gluconeogenesis (25). Although this study has no estrogen-treated group for a direct comparison, we have examined expression of these genes using real-time RT-PCR. Expression of these hepatic genes was either unchanged or was the opposite of what has been observed after estrogen treatment in rodents (24, 25, 40) despite attainment of plasma genistein and daidzein concentrations, which we have previously reported to be similar to those observed in soy formula-fed infants (28).

In summary, we have demonstrated similar biochemical and metabolic differences to those reported in breast-fed and formula-fed infants, in a neonatal piglet model of formula feeding. Moreover, Affymetrix gene array analysis of global changes in hepatic gene expression profiles appear to explain many of the observed metabolic differences associated with formula feeding and specific effects associated with consumption of soy formula. Our data are consistent with studies in rodents sug-
gesting that consumption of soy protein isolate activates the hepatic nuclear receptors LXR and PXR (51) and with previous data suggesting a lack of estrogenic action in the liver (55). Breast-feeding has been shown to have long-term benefits in later life, including reduced risks of obesity and Type 2 diabetes (29, 37). Long-term health benefits including reduced risk of cancer, cardiovascular disease, and osteoporosis have also been suggested to be associated with early soy consumption (4, 13, 15). Further investigation of the significant differences in gene expression signatures between breast- and formula-fed piglets and piglets fed milk and soy formulas is required before it can be concluded that these have a long-term impact on development and health. In particular, it will be important to determine if the effects of early diet on gene expression profiles persist beyond weaning. In this regard, such long-term programming of increased bile acid synthesis via increased CYP7A1 expression has previously been reported long-term programming of increased bile acid synthesis via expression profiles persist beyond weaning. In this regard, such long-term programming of increased bile acid synthesis via increased CYP7A1 expression has previously been reported additional studies using this pig model may provide important mechanistic information regarding the molecular mechanisms underlying these long-term health effects and developmental differences between breast- and formula-fed children.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES