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Global effects of vitamin A deficiency on gene expression in rat liver: evidence for hypoandrogenism

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Abstract

Vitamin A (retinol) metabolites are ligands for transcription factors that regulate many genes. The liver is the main storage depot for retinol and plays a role in vitamin A homeostasis. To better understand the effects of vitamin A deficiency on liver gene expression, we produced retinol deficiency in male rats by feeding a diet low in retinol for 53 days after weaning and examined the effects on gene expression in liver using Affymetrix oligonucleotide microarrays. We detected expression of 41% of the 8799 probe sets represented on the RGU-34A GeneChips. Vitamin A deficiency resulted in major changes in liver gene expression: 805 genes (22% of all genes detected) differed at $P \leq .05$ (false discovery rate <0.143). Genes involved in fatty acid metabolism, peroxisomal function, glycolysis, glutamate metabolism and the urea cycle were altered. The expression of many sexually dimorphic genes was altered toward a feminized or senescent pattern of gene expression in the liver. Retinol deficiency also produces a shift toward increased protein and fat catabolism and decreased fatty acid synthesis.
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1. Introduction

Vitamin A (retinol) plays an important role in many physiological activities through its metabolites, particularly 9-*cis* and all-*trans* retinoic acids. These retinoids are ligands for important transcription factors, including the classical all-*trans* retinoic acid receptors (RARs) and the 9-*cis* retinoic acid receptors (retinoid X receptors, RXRs). RXRs form heterodimers with many other transcription factors, including the vitamin D receptor, peroxisome proliferator-activated receptors (PPARs), thyroid hormone receptors, liver X receptor, farnesoid X receptor, pregnane X receptor and the constitutive androstane receptor [1]. These transcription factors, in turn, regulate the expression of a large number of genes. Thus, vitamin A deficiency is expected to have a major impact on gene expression.

Vitamin A deficiency has wide-ranging effects, among which are visual problems, increased inflammation, decreased growth, and, in males, decreased secretion of testosterone and blocked spermatogenesis [2]. In the liver, vitamin A deficiency has a large effect on the total microsomal content of cytochrome *P*450s [3] and is associated with decreased expression of insulin-like growth factor 1 (IGF-1), leading to decreased circulating levels of IGF-1 [4]. Decreased IGF-1 levels are also seen in starvation and calorie or diet restriction [5,6]. The effects of diet and vitamin A deficiency on IGF-1 may be related because vitamin A deficiency changes enzyme levels and histology of the small intestine accompanied by increased inflammation that can lead to decreased absorption of nutrients and decreased growth [7]. The liver is the major storage organ for retinol and

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is involved in most aspects of vitamin A homeostasis. Most retinol is stored in the hepatic stellate cells as retinyl-palmitate esters [8]. The liver expresses the retinoic acid-regulated transcription factors, with the exception of the vitamin D receptor, at high levels [9].

To understand the global effects of vitamin A deficiency on gene expression in liver, we have examined the mRNA expression profile using Affymetrix oligonucleotide microarrays.

2. Methods and materials

2.1. Induction of vitamin A deficiency in rats

Male Sprague–Dawley rats were made deficient in vitamin A by feeding a custom vitamin A-deficient diet based on the AIN93 composition (ICN, Costa Mesa, CA). They were compared with rats fed the same diet but containing 4000 IU vitamin A palmitate/kg of chow (vitamin A-sufficient). Both groups of rats were fed ad libitum. The diets were started at 21 days of age. The animals were sacrificed on the morning of Day 53 of the diet. These animals were described as part of an earlier study on the effect of vitamin A deficiency on liver alcohol dehydrogenase and alcohol metabolism [10]. Total liver mRNA was isolated from snap-frozen tissue using the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Purity of the RNA was estimated from the absorbance spectrum and by electrophoresis on 1% agarose gels.

2.2. Microarray methods

Two- and 10-μg aliquots of total liver RNA from each of eight rats (four vitamin A-deficient and four vitamin A-sufficient) were independently processed according to the Affymetrix protocol, as previously reported [11]. Each sample (16 total) was hybridized to a separate RG-U34A Affymetrix GeneChip. Two of the 2-μg aliquots were lost due to reagent failure, leaving eight GeneChips from the 10-μg samples and six from the 2-μg samples (three vitamin A-deficient and three vitamin A-sufficient). Each microarray was scanned and analyzed using Affymetrix Microarray Suite 5.0 software (MAS5; Affymetrix MicroArray Suite 5.0 User's Guide, Santa Clara, CA).

2.3. Statistical analysis

The 2- and 10-μg samples from the same animal represent technical replicates from the same RNA; technical variation due to starting with different amounts of RNA was less than the biological variation between different individual animals within a treatment group [11]. Therefore, we jointly analyzed the arrays from the 2- and 10-μg technical replicates (a total of seven arrays in each treatment group) to reduce random variations and thereby to increase the power to detect differences in expression.

To eliminate noisy data, predominantly from probe sets that were not reliably detected (those at or near background or that may reflect cross-hybridization) [11], we analyzed only those probe sets (genes) that were called "present" by MAS5 in at least half of the arrays for at least one of the conditions (vitamin A-sufficient or vitamin A-deficient) [11]. We used *t* tests to reveal significant differences between vitamin A-sufficient and vitamin A-deficient livers in the expression of the 3627 genes called present by this criterion. Log-transformed signals (expression levels) more closely approximated a normal distribution than the raw signals; therefore, we applied the Welch's approximate *t* test [12] (allowing for unequal variances) to the log(base 2) of the signals. The resultant *P* values were used to calculate false discovery rates (FDR) using the *Q* value program of Storey and Tibshirani [13,14].

Fold changes were calculated by taking the ratio of the mean of the vitamin A-deficient and the vitamin A-sufficient signal values, using the larger mean as the numerator; by convention, we show the result as negative if the mean of the vitamin-deficient samples was smaller. Genes were called turned "on" or "off" if the difference in the number of present calls between the two treatment groups was greater than 4: "on" if the vitamin A-deficient group had more present and "off" if the control group had more present.

For the closely related groups of cytochrome *P*450s and sulfotransferases, individual Affymetrix probe sets were examined using the probe match feature of NetAffx (https://www.affymetrix.com/analysis/netaffx/probematch/probe_match.affx) [15] to decide whether two or more probe sets were actually measuring the same mRNA product. For categorization of the *P*450s, Dave Nelson's *P*450 website was consulted [16].

2.4. Quantitative Real Time PCR (qRT-PCR)

Aliquots of the cDNAs synthesized for use on microarrays (from the samples that started with 10 μg of total

Table 1
qRT-PCR Primers

Gene symbol	Primer pairs	Size (bp)
Cyp2c7	F: CTTCCCTCTATCATCCATCCTGA	135
	R: GGATACACAATGACACAAGGGATC	
Cyp2c12	F: AGCAGAAATGAAGATGAGGAAACA	135
	R: TGGGCAATTAGAAGACATAAGCTGT	
DBP	F: GGCAGCCTTCCTGGAGAAG	129
	R: AAGTGGCCTCACAGTGTCCC	
GSTa2	F: CATGGCCAAGACTACCTTGTAGG	112
	R: GAGGGAAAGAGGTCAGAAGGCT	
Pklr	F: ACTTGGCTCCCACATACAAATTAGA	101
	R: GGATTCTTTGCCTGTGGGAGT	
Acaa1	F: GGAGGGATTTGTCACAGCACTT	160
	R: GCCCACTATTCATGAGGCCA	
Vim	F: GGAGTCACTTCCTCTGGTTGACAC	141
	R: GTTGCACTGAGCCTGTGCA	
GATA6	F: CTGAGCTGGTGCTGCCAAG	130
	R: AAAACAAAGGCACAGAAATCACG	
Nsep1	F: GCTTGCGGGGTTTTTATTATT	151
	R: AATCTCAACTTGACCAGATATGAAA	

F: forward, R: reverse.

Table 2
Number of probe sets with altered expression

	P		
	.05	.01	.001
Probe sets	805	427	158
FDR (%)[a]	14.3	5.4	1.4

[a] False discovery rate for the *P* value.

RNA) were used for qRT-PCR. The target mRNA sequences used by Affymetrix for probe design were used to design PCR primers, using ABI Primer Express software (version 1.1; Applied Biosystems, Foster City, CA). Primers (Table 1) were compared with the rat genome using the program BLASTn [17,18] to insure they were unique. qRT-PCR analysis was performed on the ABI7700 sequence detection system in quadruplicate for each of the eight samples, using SYBR Green PCR master mix kit (Applied Biosystems). The TaqMan rodent GAPDH (glyceraldehyde-3-phosphate dehydrogenase) control reagents (Applied Biosystems) were used to provide internal controls. For selected genes, the cDNA aliquot was diluted 200-fold; for the GAPDH control, it was diluted 2000-fold. Reactions contained 8 μL of diluted cDNA in a total volume of 20 μL. All samples were normalized to GAPDH, and the vitamin A-sufficient samples were compared with the vitamin A-deficient samples using a *t* test. Genes were selected for qRT-PCR confirmation that were of particular interest to this laboratory and because they represented a wide range of fold changes (1.3- to 59-fold).

3. Results

3.1. Global effects of retinol deficiency on rat liver

Three-week-old weanling male Sprague–Dawley rats were fed a custom vitamin A-deficient or vitamin A-sufficient diet for 53 days. This resulted in progressive vitamin A deficiency as the vitamin A stores present at birth and from maternal milk were depleted. As previously reported [10], growth of the vitamin A-deficient animals slowed after 31 days on the diet, suggesting the animals were vitamin-deficient for about 3 weeks before sacrifice. Total retinol in serum was at or near zero (0.5±1.6 μg/dL) in all the animals on the vitamin A-deficient diet and was 44±6 μg/dL for animals fed the sufficient diet [10]. Our earlier analysis of several proteins in these animals showed no difference in alcohol dehydrogenase or RXRα and an increase in RXRβ [10].

To examine the global effects of vitamin A deficiency, we measured the mRNA levels using Affymetrix RGU34A GeneChips. A total of 3627 probe sets (41%) were "present" (detected) in at least one of the two conditions. Of these, 805 (22% of all probe sets detected) differed between vitamin A-deficient and vitamin A-sufficient livers at $P \leq .05$ (FDR=14.3%) and 427 differed at $P \leq .01$ (FDR=5.4%) (Table 2). Differences as small as 12% were detected at $P \leq .01$.

Several genes involved in the metabolism and action of retinoids showed changes (Table 3). RARα2 increased 20% in the vitamin A-deficient rats; it was previously reported to be increased by 30% in vitamin A deficiency [19]. LRH1 steroidogenic receptor (Nr5a2), which heterodimerizes with RXR, was increased in the vitamin A-deficient rats (Table 4). RXR1 and RARβ were not detected. The array did not include RARγ, RXRγ or lecithin:retinol acyltransferase CES2, a carboxylesterase involved in retinyl palmitate hydrolysis, increased 1.7-fold in the vitamin A-deficient rats, while ES1, another carboxylesterase, showed a 3-fold decrease. Retinol dehydrogenases type I and II showed decreases of 1.2- and 1.7-fold, respectively. Alcohol dehydrogenase type 1 and aldehyde dehydrogenase 1A1 are involved in the metabolism of retinol to retinoic acid [20,21]. Alcohol dehydrogenase mRNA increased 1.3-fold; no change in protein levels was detected in previous studies of these same animals [10]. Aldehyde dehydrogenase 1A1 increased 1.4-fold. Recently, CYP2C39 (an alias for CYP2C7) has been found to metabolize retinoic acid to 4-hydroxyretinoic acid [22]; its expression was decreased to undetectable levels in the vitamin A-deficient animals (Table 4). CYP26, which catalyzes the oxidation of retinoic acid to 4-oxo-retinoic acid and whose expression is highly correlated with liver total retinol [23], was not a target on the array. Serum retinol binding protein increased 10% in the vitamin A-deficient rats. Cellular retinol binding protein I showed no change; it has previously been reported unchanged [24,25] or decreased 3-fold [26].

Table 3
Retinol metabolism

Gene symbol	Fold change	*P*	GenBank	UniGene	Gene	Description
Ces1	−3.08	.000000	U10697	Rn.82692	29225	carboxylesterase 1
RoDHII	−1.77	.018199	U33500	Rn.94108	299511	retinol dehydrogenase type II (RODH II)
RoDHII	−1.25	.020770	U33500	Rn.94108	299511	retinol dehydrogenase type II (RODH II)
Rbp4	1.10	.000570	K03045	Rn.108214	25703	retinol binding protein 4
Adh1	1.23	.002052	M15327	Rn.40222	24172	alcohol dehydrogenase 1
Adh1	1.35	.000784	X72792	Rn.40222	24172	alcohol dehydrogenase 1
Aldh1a1	1.44	.035243	AF001898	Rn.6132	24188	aldehyde dehydrogenase family 1, member A1
Ces2	1.69	.005728	AB010635	Rn.14535	171118	carboxylesterase 2 (intestine, liver)

Genes with *P* value of ≤.05 for the functional groups identified. Identifying data, gene symbol, GenBank, Unigene and LocusLink IDs (NCBI) and description are taken from Affymetrix annotations.

Table 4
Cytochrome *P*450s, sulfotransferases, glutathione-*S*-transferases and male senescence proteins

Gene symbol	Fold change	On/off	*P*	GenBank	UniGene	LocusLink	Description
Cytochrome P450s							
Cyp17	2.5		.00622	M21208	Rn.10172	25146	cytochrome *P*450, family 17, subfamily a, polypeptide 1
Cyp1a1	1.8		.00142	E00717	Rn.10352	24296	cytochrome *P*450, family 1, subfamily a, polypeptide 1
Cyp1a2	−1.5		.00113	K03241	Rn.5563	24297	cytochrome *P*450, family 1, subfamily a, polypeptide 2
Cyp1a2	−1.4		.00056	M26127	Rn.5563	24297	cytochrome *P*450, family 1, subfamily a, polypeptide 2
Cyp1a2	−1.4		.00035	E01184	Rn.5563	24297	cytochrome *P*450, family 1, subfamily a, polypeptide 2
Cyp2a1	1.5		.00237	M33312			rat hepatic steroid hydroxylase IIA1 (CYP2A1) gene[a]
Cyp2b15	−1.4		.00384	D17349			cytochrome *P*450 2B15 gene, exon 9[a]
Cyp2b19	−2.8		.00391	J00728	Rn.91353	29295	cytochrome *P*450, 2b19
Cyp2b19	−2.8		.00340	K00996	Rn.91353	29295	cytochrome *P*450, 2b19
Cyp2b19	−3.1		.00852	K01721	Rn.91353	29295	cytochrome *P*450, 2b19
Cyp2b19	−2.0		.02326	L00320	Rn.91353	29295	cytochrome *P*450, 2b19
Cyp2b19	−2.1		.00630	M11251	Rn.91353	29295	cytochrome *P*450, 2b19
Cyp2b19	−3.0		.00932	M13234	Rn.91353	29295	cytochrome *P*450, 2b19
Cyp2b3	−1.1		.04251	M20406	Rn.4845	286953	cytochrome *P*450IIB3
Cyp2c11	−1.4		.00715	M18363	Rn.10870	29277	cytochrome *P*450, subfamily IIC (mephenytoin 4-hydroxylase)
Cyp2c11	−1.2		.01646	J02657	Rn.10870	29277	cytochrome *P*450, subfamily IIC (mephenytoin 4-hydroxylase)
Cyp2c11	−1.6		.00333	X79081	Rn.10870	29277	cytochrome *P*450, subfamily IIC (mephenytoin 4-hydroxylase)
Cyp2c12	2.8		.00019	J03786	Rn.2586	25011	cytochrome *P*450 15-β gene (Cyp2c12)
Cyp2c12	2.6		.00012	M33550	Rn.2586	25011	cytochrome *P*450 15-β gene (Cyp2c12)
Cyp2c13	−4.6		<.00001	M82855	Rn.82715	171521	cytochrome *P*450 2c13
Cyp2c13	−6.3		<.00001	J02861	Rn.82715	171521	cytochrome *P*450 2c13
Cyp2c22	−11.3		<.00001	M58041	Rn.88025	171518	cytochrome *P*450 2c22 (Cyp2c22)
Cyp2c23	1.2		.00198	U04733	Rn.2184	83790	arachidonic acid epoxygenase
Cyp2c6	−1.4		.00063	AA945571	Rn.91122	246070	cytochrome *P*450, subfamily IIC6
Cyp2c6	−1.5		.00007	M24239	Rn.91122	246070	cytochrome *P*450, subfamily IIC6
Cyp2c6	−1.5		.00266	M14776	Rn.91122	246070	cytochrome *P*450, subfamily IIC6
Cyp2c7	−30.5	off	<.00001	AA945573			*P*450 homepage cyp2c7[a]
Cyp2c7	−59.4	off	<.00001	M18335			*P*450 homepage cyp2c7[a]
Cyp2c7	−36.8	off	<.00001	M31031			*P*450 homepage cyp2c7[a]
Cyp2c7	−378.0	off	<.00001	M14775			*P*450 homepage cyp2c7[a]
Cyp2e1	1.2		.00044	M20131	Rn.1372	25086	cytochrome *P*450, family 2, subfamily e, polypeptide 1
Cyp2e1	1.3		.00402	AF056333	Rn.1372	25086	cytochrome *P*450, family 2, subfamily e, polypeptide 1
Cyp2e1	1.4		.00004	S48325	Rn.1372	25086	cytochrome *P*450, family 2, subfamily e, polypeptide 1
Cyp3a9	−2.6	off	.01142	U46118	Rn.10489	171352	cytochrome *P*450 3A9 (Cyp3a9)
Cyp4a1	1.7		.00007	AA924267			cytochrome *P*450 4a1 from *P*450 homepage[a]
Cyp4a1	1.3		.00357	M14972			cytochrome *P*450 4a1 from *P*450 homepage[a]
Cyp4a1	1.5		.00043	X07259			cytochrome *P*450 4a1 from *P*450 homepage[a]
Cyp4f14	−1.2		.01817	M94548	Rn.5722	56266	cytochrome *P*450, subfamily IVF, polypeptide 14 (leukotriene B4 omega hydroxylase)
Sulfotransferases							
Sult1a2	−3.1		.00001	L22339	Rn.9937	65185	sulfotransferase family 1A, member 2
Sult1a2	−2.4		<.00001	L22339	Rn.9937	65185	sulfotransferase family 1A, member 2
Sult1a1	1.3		.03393	L19998	Rn.1507	83783	sulfotransferase family 1A, phenol-preferring, member 1
Sult1a1	1.4		.00093	L19998	Rn.1507	83783	sulfotransferase family 1A, phenol-preferring, member 1
Sth2	1.4		.03566	M31363	Rn.91378	24912	sulfotransferase, hydroxysteroid preferring 2
Sth2	1.5		.02056	AA818122	Rn.91378	24912	sulfotransferase, hydroxysteroid preferring 2
Sth2	1.7		.00260	D14988	Rn.91378	24912	sulfotransferase, hydroxysteroid preferring 2
Sth2	1.7		.00122	D14987	Rn.91378	24912	sulfotransferase, hydroxysteroid preferring 2
Sult1c2	1.6		.00217	AI638982	Rn.108183	171072	sulfotransferase family, cytosolic, 1C, member 2
Sult1c2	1.8		.00841	AA926193	Rn.22471	171072	sulfotransferase family, cytosolic, 1C, member 2
ST-60	1.7		.01194	D14989	Rn.40365	361510	similar to alcohol sulfotransferase (hydroxysteroid sulfotransferase) (ST-60)
ST-60	1.9		.00139	AI169695	Rn.40365	361510	similar to alcohol sulfotransferase (hydroxysteroid sulfotransferase) (ST-60)
Smp2a	2.3		.00001	AA945050	Rn.92406	24902	rat senescence marker protein 2A gene, exons 1 and 2
Smp2a	2.4		<.00001	M33329	Rn.92406	24902	rat senescence marker protein 2A gene, exons 1 and 2
Smp2a	2.5		<.00001	X63410	Rn.92406	24902	rat senescence marker protein 2A gene, exons 1 and 2

Table 4 (*continued*)

Gene symbol	Fold change	On/off	*P*	GenBank	UniGene	LocusLink	Description
Glutathione-S-transferases							
Gsta2	−2.2		.00001	K00136	Rn.120928	24422	glutathione-*S*-transferase α type 2
Gsta2	−1.5		.03417	AI235747	Rn.120928	24422	glutathione-*S*-transferase, α type 2
Gsto1	−1.6		.00002	AB008807	Rn.25166	114846	glutathione-*S*-transferase omega 1
Gsto1	−1.4		.00221	AB008807	Rn.25166	114846	glutathione-*S*-transferase omega 1
Gstt1	−1.2		.04805	X67654	Rn.11122	25260	glutathione-*S*-transferase τ 1
Gstm1	−1.1		.00053	J02810	Rn.93760	24423	glutathione-*S*-transferase μ1
Gstm1	−1.1		.00155	X04229	Rn.93760	24423	glutathione-*S*-transferase μ1
Senescence markers							
Obp3	−36.0		.00001	J00738	Rn.86413	259247	α2u globulin PGCL4
Obp3	−5.5	off	.00056	X14552	Rn.86413	259247	α2u globulin PGCL4
Obp3	−1.6		.00530	M27434	Rn.86413	259247	α2u globulin PGCL4
Rgn	−1.5		.00001	D31662	Rn.10006	25106	regucalcin

Genes with $P \leq .05$ for the functional groups identified. Identifying data, gene symbol, GenBank, Unigene and LocusLink IDs (NCBI), and description are taken from Affymetrix annotations.

On/off indicates whether gene has been turned on or off in the vitamin A-deficient rats (see Methods and Materials).

[a] For those probe sets without UniGene descriptions, descriptions are taken from the Affymetrix GeneChip description, which is from the original NCBI annotation for the sequence used for the probe set.

Differences were seen in many other groups of genes, including growth factors, nuclear receptors and transcription factors (Table 5) and fatty acid metabolism, glycolysis and gluconeogenesis, and peroxisomes (Table 6). Many cytochrome *P*450s, sulfotransferases and glutathione-*S*-transferases also differed (Table 4). The results for all 805 probe sets with $P \leq .05$ are available as supplemental data. Data for all 14 arrays are available from Gene Expression Omnibus at NCBI [27], accession numbers GSM27430 through GSM27443 and GSE1600.

3.2. Reliability of microarray data

The reliability of these microarray data was demonstrated first by the fact that redundant probe sets gave very consistent values, as shown in Tables 4–6 and Supplementary Data. Second, quantitative RT-PCR was used to confirm the observed changes in nine of the genes (Table 7) spanning a broad range of fold changes (1.3- to 59-fold). The direction and magnitude of change detected by microarrays and RT-PCR were comparable for all nine genes, and eight of the nine changes were statistically significant when tested by RT-PCR (the exception was Nsep1, in which the −1.3-fold change was consistent). Our experience is that small changes (<35%) are detected with better significance using microarrays, a linear assay, than with qRT-PCR, a logarithmic assay. The qRT-PCR showed a much greater difference for CYP2C7, a gene that was essentially turned off; in that circumstance, the exact fold change is not meaningful.

4. Discussion

One of the strengths of gene expression profiling is that patterns of change may be recognized that would be difficult to understand by analyzing individual mRNAs in isolation. This experiment revealed that vitamin A deficiency resulted in major changes in liver gene expression: 22% of all genes whose expression was detected demonstrated altered expression. We have provided a supplemental table containing all of the probe sets with a $P \leq .05$ and have deposited all of the data in the GEO database at NCBI [27], so that others may use this information. We will focus the discussion on some of the major patterns of changes that reflect interesting biological responses.

4.1. Transcription factors and nuclear receptors

Seven nuclear receptor genes showed changes; all but one were expressed at higher levels in vitamin A-deficient rats (Table 5). All of these nuclear receptors except hepatocyte nuclear factor 3γ and hepatocyte nuclear factor 4α heterodimerize with RXR. The largest increase in mRNA levels among transcription factors was observed for the albumin promoter D site binding protein (involved in circadian regulation [28]); the arrays detected a 3.5-fold increase, which was confirmed (at 5.2-fold) by qRT-PCR. Rev-ErbAα (Nr1d1), which is also involved in circadian regulation [29], was increased twofold. Changes in so many transcription factors should result in many downstream changes. Indeed, more than 22% of the genes whose expression could be detected in the liver were found to differ as a result of vitamin A deficiency.

4.2. Effects on growth hormones

We detected a 1.6-fold reduction in the level of full-length growth hormone receptor (GHR); a decrease in GHR contributes to decreased sensitivity to GH [30]. There was a 2.4-fold reduction in the GH binding protein, GHBP (a short isoform of GHR); GHBP binds to circulating GH and increases its half-life [31], so a decrease in GHBP should lower GH levels. Both of these changes are expected to reduce the physiological effects of GH. GH is the principal hormone stimulus for IGF-1 production, and most of the anabolic actions of GH are mediated by IGF-1 [6]. Decreases in circulating IGF-1 are seen in vitamin A

Table 5
Growth factors, nuclear factors and transcription factors

Gene symbol	Fold change	On/off	P	GenBank	UniGene	Gene	Description
Growth factors							
	−2.4		.00002	S49003			short isoform growth hormone receptor*
Igfals	−1.8		.03591	S46785	Rn.7327	79438	insulinlike growth factor binding protein, acid labile subunit
Igfals	−1.7		.02406	S46785	Rn.7327	79438	insulinlike growth factor binding protein, acid labile subunit
Igfals	−1.5		.01590	AA924289	Rn.7327	79438	insulinlike growth factor binding protein, acid labile subunit
Igf1	−1.7		.04611	X06107	Rn.6282	24482	insulinlike growth factor 1
Igf1	−1.6		.00835	X06107	Rn.6282	24482	insulinlike growth factor 1
Igf1	−1.2		.00987	M15481	Rn.6282	24482	insulinlike growth factor 1
Igf1	−1.1		.02932	M15481	Rn.6282	24482	insulinlike growth factor 1
Ghr	−1.6		.00104	Z83757	Rn.2178	25235	growth hormone receptor
Igfbp3	−1.5		.00810	M31837	Rn.26369	24484	insulinlike growth factor binding protein 3
Egfr	−1.3		.00901	M37394	Rn.37227	24329	epidermal growth factor receptor
Bmp6	1.2		.03375	X58830	Rn.102203	25644	bone morphogenetic protein 6
Grn	1.2		.00891	X62322	Rn.5820	29143	granulin
Grn	1.3		.03454	X62322	Rn.5820	29143	granulin
Ngfrap1	1.3		.00961	AA874794	Rn.3126	117089	nerve growth factor receptor associated protein 1
Ltbp1	1.4		.00610	AI232078	Rn.40942	59107	latent transforming growth factor β binding protein 1
Erbb3	1.4		.00631	U52530	Rn.10228	29496	v-*erb*-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
Klf9	1.5		.00176	D12769	Rn.19481	117560	Kruppel-like factor 9 (Klf9)
Ntf3	1.5		.00249	M34643	Rn.9715	81737	neurotrophin 3
Ntf3	1.7		.02035	E03082	Rn.9715	81737	neurotrophin 3
Igfbp2	2.3		.01458	J04486	Rn.6813	25662	insulinlike growth factor binding protein 2
Nuclear receptors and transcription factors							
Nr1h4	−1.4		.00076	U18374	Rn.42943	60351	nuclear receptor subfamily 1, group H, member 4
Rara	1.2		.02082	U15211	Rn.91057	24705	retinoic acid receptor α
Nfkb1	1.3		.01456	L26267	Rn.2411	81736	nuclear factor kappa B p105 subunit
Nr5a2	1.3		.01538	AB012960	Rn.42941	60349	nuclear receptor subfamily 5, group A, member 2
Hnf3g	1.3		.02570	AB017044	Rn.10949	25100	hepatocyte nuclear factor 3 γ
Hnf3g	1.3		.04580	AB017044	Rn.10949	25100	hepatocyte nuclear factor 3 γ
Hnf4a	1.3		.00228	X57133			hepatocyte nuclear factor 4α*
Hnf4a	1.3		.01561	D10554	Rn.44442	25735	hepatocyte nuclear factor 4α
Gata6	1.4		.00012	L22760	Rn.8701	29300	GATA binding protein 6
Nrbf1	1.5		.00078	AB015724	Rn.15375	29470	nuclear receptor binding factor 1
Nr1d2	1.7		.01934	U20796	Rn.10055	259241	nuclear receptor subfamily 1, group D, member 2
Nr1d1	1.8		.00790	M25804	Rn.29848	252917	nuclear receptor subfamily 1, group D, member 1
Nr1d1	2.8	on	.00398	M25804	Rn.29848	252917	nuclear receptor subfamily 1, group D, member 1
Dbp	2.9	on	.02372	J03179	Rn.11274	24309	D site albumin promoter binding protein
Dbp	3.5	on	.00742	J03179	Rn.11274	24309	D site albumin promoter binding protein

* For probe sets without a Unigene number, the description is taken from the GenBank sequence used for the probe set.

deficiency [4], malnutrition [32] and aging humans [33]. GH treatment in humans increases IGF-1, IGFBP-3 and IGFALS and decreases IGFBP-1 and IGFBP-2 [34]. The vitamin A-deficient rats have decreased expression of IGF-1, IGFBP3 and IGFALS and increased IGFBP2. Thus, the changes we detected are consistent with reduced effects of GH due to decreases in GHR and GHBP.

Because the decrease in IGF-1 and some of the metabolic effects detected in the vitamin A-deficient rats are also seen in starvation, we compared our results to a microarray study of starvation in 8- to 15-week-old male 129/SV mice [35]. Both the vitamin A-deficient rats (our study) and mice starved for 24 or 48 h showed decreases in mRNA levels for enzymes involved in fatty acid synthesis (e.g., fatty acid synthase) and increases in fatty acid oxidation (e.g., enoyl-coenzyme A hydratase). The starved mice had decreases in fatty acid binding proteins that were not seen in the vitamin A-deficient rats. There were larger increases (>2-fold) in urea cycle enzymes in the starved mice than in the vitamin A-deficient rats (≤1.5-fold). The down-regulation of genes involved in cholesterol synthesis seen in the starved mice was not seen in the vitamin A-deficient rats, but there was a similar decrease in CYP17A1 (involved in the synthesis of DHEA from cholesterol). Among the 12 *P*450s with altered expression in starved mice that were also available on the rat array, three changed in the same direction, one differed in the opposite direction, four had no significant change and four were not detectable (absent). The dramatic changes that we saw in sex-specific or sex-dominant Cyps (e.g., CYP2C11, CYP2C13, CYP2C7) were not reported in the starved mice. Starved mice and vitamin A-deficient rats showed similar changes in IGF-1 and associated proteins: decreases in IGF-1, IGFBP3 and IGFALS and increases in IGFBP1 and IGFBP2. No changes in GHR or GHBP were

Table 6
Metabolism

Gene symbol	Fold change	On/off	*P*	GenBank	UniGene	LocusLink	Description
Fatty acid metabolism							
Scd1	−10.0	off	.00064	AI175764	Rn.1023	246074	stearoyl-coenzyme A desaturase 1
Scd1	−7.3		.00001	J02585	Rn.1023	246074	stearoyl-coenzyme A desaturase 1
Me1	−2.1		.01134	M26594	Rn.3519	24552	malic enzyme 1
Me1	−1.8		.00846	AI171506	Rn.3519	24552	malic enzyme 1
Acsl4	−1.9		.00024	AI236284	Rn.87821	113976	acyl-coenzyme A synthetase long-chain family member 4
Acsl4	−1.5		.00006	D85189	Rn.87821	113976	acyl-coenzyme A synthetase long-chain family member 4
Fasn	−1.9		.00044	M76767	Rn.9486	50671	fatty acid synthase
Acly	−1.8		.01037	J05210	Rn.29771	24159	ATP citrate lyase
Acly	−1.7		.03814	L27075	Rn.29771	24159	ATP citrate lyase
Acly	−1.5		.00278	J05210	Rn.29771	24159	ATP citrate lyase
Lrp2	−1.4		.01419	L34049	Rn.26430	29216	low density lipoprotein receptor-related protein 2
Apoa4	−1.2		.00593	M00002	Rn.15739	25080	apolipoprotein A-IV
Apoe	1.1		.02984	S76779	Rn.32351	25728	apolipoprotein E
Ucp2	1.2		.03742	AB010743	Rn.13333	54315	uncoupling protein 2
Lcat	1.3		.00214	X54096	Rn.10481	24530	lecithin cholesterol acyltransferase
Slc27a2	1.3		.00002	D85100	Rn.3608	65192	solute carrier family 27 (fatty acid transporter), member 2
Decr1	1.3		.02800	D00569	Rn.2854	117543	2,4-dienoyl coenzyme A reductase 1, mitochondrial
Scarb1	1.1		.02052	AA874843	Rn.88169	25073	scavenger receptor class B, member 1
Scarb1	1.3		.00261	D89655	Rn.88169	25073	scavenger receptor class B, member 1
Decr1	1.4		.02813	D00569	Rn.2854	117543	2,4-dienoyl coenzyme A reductase 1, mitochondrial
Acox2	1.5		.01513	X95189	Rn.10622	252898	acyl-coenzyme A oxidase 2, branched chain
Cpt1a	1.6		.01187	L07736	Rn.2856	25757	carnitine palmitoyltransferase 1, liver
Cd36	1.7	on	.00366	AA946368	Rn.3790	29184	cd36 antigen
Cd36	1.8		.00445	AF072411	Rn.3790	29184	cd36 antigen
Cd36	1.8		.00470	AF072411	Rn.3790	29184	cd36 antigen
Cd36	1.8		.00533	AA925752	Rn.102418	29184	cd36 antigen
	1.5		.00589	J02597			apolipoprotein A-I gene*
Apoa1	1.8		.01002	M00001	Rn.10308	25081	apolipoprotein A-I
Scd2	3.6	on	.00409	AA875269	Rn.83595	83792	stearoyl-coenzyme A desaturase 2
Scd2	4.0		.00283	M15114	Rn.83595	83792	stearoyl-coenzyme A desaturase 2
Peroxisomal							
Acaa1	−2.4		<.00001	J02749	Rn.47075	24157	acetyl-coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-coenzyme A thiolase)
Decr2	−1.4		.00858	AF044574	Rn.7879	64461	2-4-dienoyl-coenzyme A reductase 2, peroxisomal
Decr2	−1.2		.00709	AF044574	Rn.7879	64461	2-4-dienoyl-coenzyme A reductase 2, peroxisomal
Pex14	−1.3		.00072	AB017544	Rn.7844	64460	peroxisomal biogenesis factor 14
Acaa1	−1.2		.00352	J02749	Rn.8913	24157	acetyl-coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-coenzyme A thiolase)
Pxmp2	1.3		.00015	X70223	Rn.10292	29533	peroxisomal membrane protein 2
Slc16a7	1.3		.00352	U62316	Rn.10524	29735	solute carrier family 16 (monocarboxylic acid transporters), member 7
Ehhadh	1.5		.01051	K03249	Rn.3671	171142	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
Ech1	1.5		.01954	U08976	Rn.6148	64526	enoyl coenzyme A hydratase 1, peroxisomal
Glucose metabolism							
Pklr	−2.5		.00029	X05684	Rn.48821	24651	pyruvate kinase, liver and RBC
Pdk4	−2.3		.04435	AF034577	Rn.30070	89813	pyruvate dehydrogenase kinase, isoenzyme 4
Tkt	−1.7		.00003	U09256	Rn.5950	64524	transketolase
Tkt	−1.7		.00276	AI059508	Rn.5950	64524	transketolase
Pgk1	−1.4		.00058	M31788	Rn.108127	24644	phosphoglycerate kinase 1
	−1.3		.00018	M54926			rat lactate dehydrogenase A mRNA*
	−1.3		.00063	M86235			M86235 rat ketohexokinase*
Pgm1	−1.3		.01446	U20195	Rn.9970	24645	phosphoglucomutase 1
Pygl	−1.3		.03745	X04069	Rn.21399	64035	liver glycogen phosphorylase
Gckr	−1.2		.00700	AA945442	Rn.7863	25658	glucokinase regulatory protein
Taldo1	−1.2		.01105	AA799452	Rn.3136	83688	transaldolase 1
Eno1	−1.2		.03856	X02610	Rn.4236	24333	enolase 1 α
Pgm1	−1.2		.03256	L11694	Rn.9970	24645	phosphoglucomutase 1
Gys2	1.1		.04069	J05446	Rn.2906	25623	glycogen synthase 2
Aldob	1.2		.00838	X02284	Rn.98207	24190	aldolase B

(*continued on next page*)

Table 6 (*continued*)

Gene symbol	Fold change	On/off	*P*	GenBank	UniGene	LocusLink	Description
Glucose metabolism							
Pfkm	1.3		.00210	U25651	Rn.11004	65152	phosphofructokinase, muscle
Ppp2r2a	1.3		.01785	M83298	Rn.81155	117104	protein phosphatase 2 (formerly 2A)
Ppp1r2	1.3		.01258	S79213	Rn.4016	192361	protein phosphatase 1, regulatory (inhibitor) subunit 2
Aldoa	1.3		.00140	M12919	Rn.1774	24189	aldolase A
Aldoa	1.5		<.00001	M12919	Rn.1774	24189	aldolase A
Aldoa	1.5		.00118	AA924326	Rn.1774	24189	aldolase A
Glutamate metabolism							
Glul	−1.7		.00513	AA852004	Rn.2204	24957	glutamine synthetase 1
Glul	−1.6		.00168	M91652	Rn.2204	24957	glutamine synthetase 1
Glul	−1.5		.00749	M91652	Rn.2204	24957	glutamine synthetase 1
Aldh5a1	−1.4		.00357	L34821	Rn.10070	29505	aldehyde dehydrogenase family 5, subfamily A1
Gls2	1.4		.00041	J05499	Rn.10202	192268	glutaminase 2 (liver, mitochondrial)
Urea cycle							
Oat	−2.2		.00013	M93297	Rn.1430	64313	ornithine aminotransferase
Oat	−1.8		.00160	AA893325	Rn.1430	64313	ornithine aminotransferase
Cps1	1.2		.00397	M11710	Rn.53968	24956	carbamoyl-phosphate synthetase 1, mitochondrial
Asl	1.2		.02597	D13978	Rn.64591	59085	argininosuccinate lyase
Otc	1.3		.04240	K03041	Rn.2391	25611	ornithine transcarbamylase
Ass	1.4		.00034	X12459	Rn.5078	25698	arginosuccinate synthetase
Arg1	1.5		.00036	J02720	Rn.9857	29221	arginase 1

reported in the mice, in contrast to our data. Starvation has been shown to lead to decreased GHR and GHBP expression, GH resistance and decreased total body protein synthesis [34]. While some of the catabolic effects that we found due to vitamin A deficiency may be explained by GH resistance and subsequent decrease in IGF-1, the effects on sexually dimorphic gene expression do not appear to be the result of malnutrition.

4.3. Cytochrome P450s and feminization of gene expression

Eleven of the 18 *P*450s (measured by 38 probe sets) that differed were decreased in the vitamin A-deficient animals (Table 4), consistent with an overall decrease in cytochrome *P*450s that was reported earlier [3]. CYP2E1 is up-regulated, which also occurs with chronic alcohol consumption [36]. We noticed that the pattern of changes in *P*450s was related to sex differences in their expression. Male-specific *P*450s (CYP2C11, CYP2C13 and CYP2C22) were all decreased in the vitamin A-deficient animals. Female-specific CYP2C12 was increased about 2.6-fold. Three other groups of genes known to have sexually dimorphic expression patterns are male senescence markers, sulfotransferases and glutathione-*S*-transferases (Table 4). Male-specific protein α2u globulin (Table 4), a major urinary protein involved in transport of pheromones to the urine, was turned off. Male-specific hydroxy-2-acetylaminofluorene sulfotransferase (Sult1a2, Table 4) [37] was decreased approximately threefold. All other sulfotransferases that changed were increased. There were significant decreases in glutathione-*S*-transferases GSTα type 2 and GSTμ, which are male-dominant [38]. This overall pattern of change across several groups of genes suggests that vitamin A deficiency leads to a feminization of gene expression in liver.

This pattern of changes in the vitamin A-deficient animals also resembles the effects of aging mediated by GH secretion patterns. Young adult male rats show episodic

Table 7
Comparison between qRT-PCR and microarray results

Gene symbol	qRT-PCR		Array		Description
	*P**	Fold change	*P***	Fold change	
Dbp	.0011	5.2	.0074	3.5	D site albumin promoter binding protein
Cyp2c12	.0000	3.8	.0002	2.8	cytochrome *P*450 2C12
Vim	.0042	1.8	.0002	1.7	vimentin
Gata6	.0064	1.5	.0001	1.4	GATA-binding protein 6
Nsep1	.5918	−1.3	.0012	−1.3	nuclease sensitive element binding protein 1
GSTa2	<.0001	−2.2	<.0001	−2.2	glutathione-*S*-transferase α type 2
Acaa1	<.0001	−2.5	<.0001	−2.4	acetyl-coenzyme A acyltransferase 1 (peroxisomal)
Pklr	<.0001	−3.1	.0003	−2.5	pyruvate kinase, liver and RBC
Cyp2c7	<.0001	−555	<.0001	−59.4	cytochrome *P*450 2C7

* *P* value for *t* test assuming unequal variance for qRT-PCR data.
** *P* value for *t* test using log transform of Affymetrix signal assuming unequal variance.

spikes of GH every 3–3.5 h with undetectable levels between spikes, whereas females have a more continuous secretion of GH with more pulses at lower amplitude and GH detectable between pulses [39]. Middle-aged males and senescent males have a more feminized pattern of GH secretion [39,40], which leads to the feminization of gene expression. Several sex-specific or sex-dominant *P*450 cytochromes are known to be regulated by sex-specific GH secretion patterns [39–42]. Reduced expression of CYP2C7, CYP2C11 and CYP2C13, reported for middle-aged male rats [40], was observed in the vitamin A-deficient rats (Table 4). Sex-specific patterns of GH are also known to regulate a group of sulfotransferases (Table 4), Sult1a2, Smp2a and St-60 [37] and fatty acid translocase (Table 6) [43]; these were among the genes whose expression pattern changed in this experiment. We observed decreases in α2u globulin and regucalcin and increases in expression of sulfotransferases Smp2a and Sth2 and cytochromes CYP2A1 and CYP2C12, characteristic of senescent males [39,44] (Table 4); CYP2C6, for which we saw a 50% decrease, was the only exception.

This feminization of liver gene expression might be secondary to a reduction of testosterone and alteration of GH secretion pattern. Vitamin A deficiency in male rats reduces the basal secretion of testosterone, blocks spermatogenesis and results in atrophy of accessory sex organs and the growth of female-type fur in male rats [2]. Although vitamin A deficiency has been shown to decrease serum levels of GH [45], its effect on the secretory pattern of GH does not appear to have been studied. The pattern of circulating GH levels, especially interpeak levels, is responsible for sexually dimorphic expression mediated by the Jak/Stat pathway [46]. Changes in male-specific gene expression such as in CYP2C11 may be due to desensitization of the JAK-Stat system in vitamin A-deficient livers [47]. A recent microarray study by Ahluwalia et al. [42] reported 71 genes that have sexually dimorphic expression patterns in rat liver are affected by introducing a feminized pattern of GH expression into male rats. Only 34 of these genes were detected by the RGU34A GeneChip, and of these, we observed changes in the same direction in about 60% and no observable changes in another 30%. There were three genes (10%) for which we saw significant changes in the opposite direction: pancreatic secretory trypsin inhibitor (1.3), CYP2C7 (off) and Acyl-coenzyme A-synthetase 4 (−1.9). These genes may be more directly affected by vitamin A status. CYP2C7 (alias for CYP2C39) metabolizes retinoic acid [22].

4.4. Effects on metabolism

Since retinoic acid can modify the action of the PPARα on some promoters, it was interesting that a cluster of genes affected by vitamin A deficiency are known to be controlled by PPARα or to be inducible with peroxisome proliferators (Table 6). Of nine mRNAs thought to be regulated by PPARα that were changed in this experiment, seven were higher (cytochrome *P*450 4A1, ApoAI, uncoupling protein-2, peroxisomal bifunctional enzyme, carnitine palmitoyltransferase 1 and fatty acid translocase) and two were lower (pyruvate dehydrogenase kinase 4 and peroxisomal ketoacyl-coenzyme A thiolase) in the vitamin A-deficient animals. This is counterintuitive, as the PPARα/RXR heterodimer in some (but not all) cases is stimulated by the presence of 9-*cis* retinoic acid plus the PPARα ligand. However, other research has shown that vitamin A-deficient rats can still respond to peroxisome proliferators [48]. Hypovitaminosis A has been shown to be associated with increased plasma free fatty acids (FFAs) [49]. An induction of PPARα-regulated genes would be consistent with increased plasma FFA. Although PPARα has been shown to be more highly expressed in males than females, it is affected by sex hormones and not by sexually dimorphic GH patterns [50].

Levels of sterol regulatory element binding protein-1 (SREBP-1) mRNA were unchanged. On the other hand, four of the five genes regulated by SREBP-1 were decreased in the vitamin A-deficient animals: fatty acid synthase, SCD1, ATP citrate lyase and malic enzyme (Table 6); scavenger receptor B1 was the exception. SCD1 is the primary stearoyl-coenzyme A desaturase in the liver of rats [51] but in the vitamin A-deficient rats SCD1 was turned off and SCD2 was turned on; these two SCD genes have different 5′-regulatory regions [51]. These changes indicate a shift from fatty acid synthesis to β-oxidation, opposite to the change in alcoholic liver disease that produces fatty liver [52].

Vitamin A plays a role in the regulation of carbon flux through the glycolytic and gluconeogenic pathways. Vitamin A deficiency decreases hepatic gluconeogenesis [53]. There were a number of changes to genes in these pathways (Table 6); Aldolase A mRNA was increased 30% to 50%, and large decreases were seen for pyruvate kinase mRNA (−2.6-fold) and pyruvate dehydrogenase kinase 4 (−2.3-fold). We did not detect changes in phosphoenol pyruvate carboxykinase or 6-phosphofructose-2-kinase/2,6-bisphosphatase, despite earlier reports that they are lower in vitamin A-deficient mice [54].

Genes involved in glutamine synthesis were down-regulated in vitamin A-deficient rat livers (Table 6). Glutamine synthetase, involved in detoxification of ammonia, was decreased 1.6-fold. Ornithine aminotransferase mRNA was reduced 2.2-fold (55%), similar to an earlier report that it was decreased 70% in vitamin A deficiency [55]. In contrast, glutaminase was increased. The mRNAs for 5 enzymes in the urea cycle (Table 6) were increased: mitochondrial carbamoyl phosphate synthetase (1.2-fold), ornithine transcarbamylase (1.3-fold), argininosuccinate synthetase (1.4-fold), argininosuccinate lyase (1.2-fold) and arginase (1.5-fold).

Recently Deaciuc et al. [56] performed a microarray study of male mice receiving intragastric infusion of ethanol for 28 days compared with pair-fed controls. Only a few of the genes that responded to ethanol responded to vitamin A deficiency. SCD1 and fatty acid synthase were

down-regulated in both conditions, but in contrast to our observed decreases in GSTs in vitamin A-deficient rats, ethanol led to marked increases in GSTs [55].

The large number of changes that result from vitamin A deficiency are likely due to the reduced availability of the receptor ligand retinoic acid, amplified by changes in the expression of several transcription factors and in the nutritional and endocrine status of the animals. Broadly, the microarray data on gene expression showed that vitamin A deficiency is a catabolic state that might in part be due to decreased nutrient availability [7]. Increased plasma FFAs stimulates PPARα-controlled pathways, which generally promote fat oxidation or export as lipoproteins, and inhibits SREBP-1-regulated genes, which are involved in fat synthesis; both of these changes were detected in the vitamin A-deficient rats of this study (Table 6). The vitamin A-deficient rats exhibited dramatic changes in sexually dimorphic gene expression, which do not appear to be a result of decreased nutrition and may be related to post-GHR Jak-Stat insensitivity to GH seen in vitamin A-deficient animals [47].

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2005.08.006.

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