Disruption of lipid metabolism in the liver of the pregnant rat fed folate-deficient and methyl donor-deficient diets

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The importance of folic acid and the methionine cycle in fetal development is well recognised even though the mechanism has not been established. Since the cycle is active in the maternal liver, poor folate status may modify hepatic metabolism. Pregnant rats were fed diets deficient in folic acid (−F) or in three key methyl donors, folic acid, choline and methionine (−FLMLC) and the maternal liver was analysed on day 21 of gestation. Two-dimensional gel electrophoresis of soluble proteins identified differentially abundant proteins, which could be allocated into nine functional groups. Five involved in metabolic processes, namely, folate/methionine cycle, tyrosine metabolism, protein metabolism, energy metabolism and lipid metabolism, and three in cellular processes, namely, endoplasmic reticulum function, bile production and antioxidant defence. The mRNA for sterol regulatory element-binding protein-1c and acetyl-CoA carboxylase-1 (fatty acid synthesis) were decreased by both −F and −FLMLC diets. The mRNA for PPARα and PPARγ and carnitine palmitoyl transferase (fatty acid oxidation) were increased in the animals fed the −FLMLC diets. Changes in the abundance of proteins associated with intracellular lipid transport suggest that folate deficiency interferes with lipid export. Reduced fatty acid synthesis appeared to prevent steatosis in animals fed the −F diet. Even with increased oxidation, TAG concentrations were approximately three-fold higher in animals fed the −FLMLC diet and were associated with an increase in the relative abundance of proteins associated with oxidative stress. Fetal development may be indirectly affected by these changes in hepatic lipid metabolism.

Methionine: Choline: Folic acid: Endoplasmic reticulum

There is overwhelming evidence to show that folic acid supplements during pregnancy are efficacious in reducing the risk of neural tube defects (NTD) in the fetus. However, numerous studies of folate levels have failed to establish a connection between folic acid metabolism in the fetus and the risk of NTD[1,2]. Moreover, the frequency of NTD is not increased following the inactivation of enzymes essential for folate metabolism in the fetus[3]. An adequate intake of folic acid during the weeks just before and after conception also improves the overall outcome[4], notably, increasing infant birth weight[5] and reducing the incidence of spontaneous abortion and pre-term delivery[6]. However, as with NTD, there is no clear evidence to link impaired fetal growth directly to folate deficiency in fetal tissues. These observations suggest that some of the broad benefits of improved folate status could be the indirect consequences of improvements in maternal metabolism.

Derivatives of folic acid play an important role in one-carbon transfer reactions, supplying methyl groups to complete the methionine cycle. Under some circumstances choline and methionine also donate methyl groups and their availability in the diet modifies the requirement for folic acid in one-carbon transfers. Before conception, the main consumer of methyl groups supplied by the methionine cycle is believed to be the hepatic enzyme phosphatidyl ethanolamine methyl transferase (PEMT)[7]. Therefore, folate deficiency during pregnancy may alter hepatic function and disturb maternal metabolic homeostasis, indirectly affecting fetal development. In the present study, a combination of two approaches has been used to examine the liver of pregnant rats fed one of two experimental diets; either a diet deficient in folic acid (−F), or one deficient in all three key methyl donors, folic acid, choline and methionine (−FLMLC). First, we analysed the soluble protein fraction from the maternal liver on day 21 of gestation (term is 22·5 d in the rat) by two-dimensional electrophoresis. Since the cytosol is in communication with many of the intracellular compartments, this fraction gives an overall picture of the changes. Second, we carried out a targeted analysis of hepatic mRNA coding for the enzymes that control the synthesis of fatty acids (acetyl CoA carboxylase-1), fatty acid oxidation (carnitine palmitoyl transferase-1) and the transcriptional regulators that control them (PPAR, CCAAT enhancer binding protein and sterol response element-binding protein-1c (SREBP-1c)). We have also investigated the expression of gadd153 and p57 to evaluate the extent of metabolic stress.

Methods

Experimental diets

The experimental diets were based on the AIN-76 formula[8] and contained 90 g casein/kg supplemented with a mixture of synthetic amino acids equivalent to those found in a further 90 g/kg casein as described previously[9]. Folic acid was omitted from the −F and −FLMLC diets. No additional methionine was added to the −FLMLC diet (total methionine concentration 2·3 mg methionine per kg diet compared with 5·6 mg methionine per kg diet in the control) and choline chloride was reduced to 0·1 % w/w (compared with 0·2 % w/w in the control).

Animals

All experimental procedures were approved by the ethical review committee of the Rowett Research Institute and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986. Female rats of the Rowett Hooded strain bred in the institute were allocated to three groups of eight animals. The animals were approximately 8 to 10 weeks of age at the start of the experiment with a mean body weight of 208 g. There were no significant differences between the weights of animals in the different treatment groups when the experiment commenced. Animals were housed on sawdust bedding and no steps were taken to prevent coprophagy. Three groups of animals were fed control, −F or −FLMLC diets. The animals were offered the experimental diets for a 2-week adaptation period and then were mated with males of the same strain. The day on which a vaginal plug was detected was denoted day 0. The female rats were maintained on their corresponding diets until day 21 of gestation, when they were anaesthetised by halothane inhalation and killed by exsanguination. The fetuses were rapidly removed, weighed and killed by decapitation. Samples of the maternal liver were frozen in liquid N as soon as possible and subsequently stored at −80°C until required. Plasma was prepared from samples of trunk blood and alanine aminotransferase and aspartate aminotransferase were analysed using a Konelab selective chemistry analyser (Labmedics Ltd, Salford, Manchester, UK).

Proteomics

Pieces of frozen maternal liver from six pregnant animals chosen randomly from each group were homogenised in buffer containing protease inhibitors as described previously[10]. The homogenate was centrifuged for 30 min at 100 000 g at 4°C (Beckman TL-100 centrifuge; Beckman Coulter Ltd, High Wycombe, Bucks, UK). Proteins in the supernatant (300 μg from each extract) were separated in the first dimension on BioRad immobilized pH gradient strips (pI 3–10; BioRad, Hemel Hempstead, UK)[11]. Following equilibration with the second dimension buffer, which contained 135 mM-iodoacetamide, proteins were separated on an 18 × 18 cm SDS polyacrylamide gel at 200 V for 9·5 h[11]. Molecular weights were determined with a Bio-Rad Precision Plus Protein Mr standard. Gels were stained with colloidal coomassie brilliant blue, dried and scanned as previously described[11]. The gel images were analysed (PDQuest v7 BioRad) and spots of interest were excised from the gels using a robotic spot cutter (BioRad). Protein identities were determined by MALDI-TOF (Voyager-DE PRO; Applied Biosystems, Warrington, Cheshire, UK) or by liquid chromatography/MS/MS (Q-trap; Applied Biosystems) MS of trypsin-digested protein spots as previously described[11]. The mass spectra of spots that were significantly different were corrected for isotope abundance and the resulting peptide mass list profiles were analysed using the Matrix Science 'Mascot' web tool (http://www.matrixscience.com). The Mascot database search criteria allowed one missed cleavage, carbamidomethyl modification of cysteine; partial oxidation of methionine, a charged state of MH + and positive identities of at least 20 % matched peptides covering at least 10 % of the protein sequence. Positive matches with proteins in the database were assigned at a probability value of $P < 0.05$.

TAG analysis

Approximately 0·2 g tissue was homogenised in 1 ml ice cold 0·145 M-NaCl. The homogenate was extracted with 10 ml chloroform:methanol (2:1). The chloroform phase was transferred to a clean tube and the residue was extracted with a further 3 ml chloroform. The extracts were combined, the chloroform was removed by evaporation and the lipids were dissolved in 5 ml absolute ethanol. TAG in 10 μl samples of the ethanol suspension were measured by adding 0·25 ml reagent (ThemoElectron Triglyceride Kit; Lab Medics Ltd), incubating for 20 min at room temperature and measuring the absorbance at 510 nm. TAG concentrations in the samples were determined from a standard curve.

Real Time PCR

Total RNA was extracted using the Trizol reagent (Sigma, Poole, Dorset, UK) as described previously[12]. Samples of 50 ng total RNA were reverse transcribed using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems) primed with random hexamers. The levels of cDNA relative to the 18S ribosomal RNA were measured using the SYBR Green real time PCR kit (Applied Biosystems). The primer sequences used are described in Table 1. The identity of the products was confirmed by sequencing and the response was linear over the range measured. Relative target quantity was calculated from the standard curve and the results expressed as the ratio of the product relative to the product from the 18S rRNA.

Statistical analysis

Data are presented as means with their standard errors of the mean. Scans of the proteomic gels (six animals per treatment) were normalised using PDQuest software to determine the mean corrected spot density. The PDQuest statistical analysis (Student's t test) was performed by pairwise comparisons of each spot on the control gels with either the −F or the −FLMLC gels. The spot densities of proteins identified in this preliminary screen were subsequently analysed by one-way ANOVA followed by Fischer's multiple comparison test (Genstat 7 statistical package; Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden,

Table 1. Primers used for PCR analysis*

Gene	Forward primer	Reverse primer	Product size
Acc	CAACGCCTTCACACCACCTT	AGCCCATTACTTCATCAAAGATCCT	104
L-CPT-1	CGGTTCAAGAATGGCATCATC	TCACACCCACCACCACGATA	75
PPAR-α	CTATGGAGTCCACGCATGTGA	TACGCCAGCTTTAGCCGAAT	68
PPARγ	CACAATGCCATCAGGTTTGG	CAGCTTCTCCTTCTCGGCCT	50
SREBP-1c	GGAGCCATGGATTGCACATT	CAAATAGGCCAGGGAAGTCAC	75
C/EBP-α	TGCGCAAGAGCCGAGATAA	TGGTCAACTCCAACACCTTCTG	69
Gadd153	CCACCACACCTGAAAGCAGAA	CACTGTCTCAAAGGCGAAAGG	78
p57	CCGGTTCCTGCTACATGAATG	GCAGCGGACGATGGAAGA	64
18 s	CATTCGTATTGCGCCGCTA	ATGCTTTCGCTCTGGTTCGT	66

*For details of procedures, see Methods.
Acc, acetyl CoA carboxylase; L-CPT-1, carnitine palmitoyl transferase; SREBP-1c, sterol response element-binding protein 1c; C/EBP-α, CCAAT enhancer binding protein α.

Herts., UK). The gene expression data were analysed by one-way ANOVA followed by Fischer's multiple comparison test (Genstat).

Results

Maternal growth

The growth of the animals has been described previously[9]. Briefly, the weight gain of animals fed the −F diet during gestation was not different from the control group, whereas the animals fed the −FLMLC diet gained approximately 15 % less than animals in the control group. Food intake during the last week of gestation did not differ significantly between the groups. The number of fetuses was not affected by the diets. Fetuses of dams fed the −F diet were approximately 18 % heavier (4·84 (SEM 0·07) g) than the controls (4·10 (SEM 0·03) g), while those from dams fed the −FLMLC diet were approximately 10 % smaller than the controls (3·71 (SEM 0·07) g). There were no gross abnormalities in either restricted group. The livers of dams in the −F group (10·1 (SEM 0·6) g) and −FLMLC group (9·1 (SEM 0·4) g) were significantly smaller ($P=0·002$) than those of animals in the control group (11·8 (SEM 1·2) g). The metabolic changes observed in these animals have been described previously[9]. Hepatic folate was reduced by approximately 80 % in animals fed −F or −FLMLC diets. Hepatic phosphocholine stores were reduced by approximately 30 % (−F) and 60 % (−FLMLC) when compared with the control. Plasma homocysteine, glycine, serine and threonine concentrations in the maternal plasma of animals fed the −F diet were higher than the control and this was further exacerbated by the −FLMLC diet[9].

Proteomic analysis of soluble proteins

Two-dimensional gel electrophoresis separated approximately 900 proteins in the soluble fraction of the female rat liver at day 21 of gestation. Comparisons of the relative abundance of proteins from the liver of animals fed the control diet with those fed the −F diet showed that eighteen proteins were up regulated and sixteen were down regulated (Student's *t* test $P<0·05$). Comparisons of the patterns from animals fed the −FLMLC diet with the control showed that twenty-six proteins were up regulated and sixteen were down

regulated ($P<0·05$). A further thirteen proteins were identified as changed ($P<0·05$) in a comparison of the soluble hepatic proteins in livers from animals fed −F and −F M LC diets.

All of the proteins identified in the initial analysis were sequenced by MS and their identities are shown in Table 2. The proteins were allocated to different groups depending on their functions. Proteins associated with metabolic functions are shown in Table 3. They include proteins associated with folate/methionine metabolism, protein and amino acid metabolism, tyrosine metabolism, energy metabolism and lipid metabolism. There were also a number of proteins associated with cellular functions and these are shown in Table 4. These proteins could be allocated into groups associated with the endoplasmic reticulum, bile production and oxidative stress. There were also a small number of proteins that could not be allocated to specific functions.

TAG in the maternal liver

There were no differences in maternal serum TAG concentrations (Fig. 1). The TAG content of the maternal liver of animals fed −F diets was not significantly different from the controls. In contrast, the hepatic TAG content of dams fed the −FLMLC diet was increased by three-fold compared with the controls. The serum alanine aminotransferase and aspartate aminotransferase activities were not different from the controls in either −F or −FLMLC diet groups (data not shown).

Gene expression in the maternal liver

The relative expression of a number of mRNA associated with lipid metabolism in the maternal liver is shown in Table 5. The hepatic acetyl CoA carboxylase-1 mRNA levels was decreased by approximately 25 % in the livers of animals fed the −F and −FLMLC diets when compared with the controls. At the same time, there was an increase of approximately 50 % in the relative expression of carnitine palmitoyl transferase-1 in animals fed the −FLMLC diet. The mRNA for PPARα and PPARγ was increased by approximately 25 % in the livers of animals fed the −FLMLC diet. The mRNA for SREBP-1c was reduced by approximately 50 % in the animals fed −F and −FLMLC diets. There was no change in the relative expression of CCAAT enhancer binding protein-α, gadd153 or p57.

Table 2. Sizes and identities of differentially abundant proteins†

PI number	p (peptide mass fingerprint MALDI)	p (Ion Score, LCMSMS)	Entrez code	Mr	Predicted Mr	pI	Predicted pI
206		1·1E-15	BAA04261	28·9	28·6	5·5	4·8
606		2·0E-04	AAH62395	52·9	48·1	5·1	4·3
1114		3·8E-13	A57716	21·9	21·9	5·9	5·3
1306*	0·05	2·1E-07	AAH86581	32·0	35·8	5·8	5·2
1308		1·6E-27	BAA90692	33·5	33·9	5·9	5·3
1509*	0·00069		AAH60597	48·4	50·2	5·7	4·8
2005		7·7E-14	AAD47293	14·9	15·8	6·1	5·1
2203			AAH78867	29·1	30·9	6·2	5·4
2401		2·8E-17	Q63041	38·7	55·1	6·0	4·9
2407	3·80E-06		CAA06313	42·0	39·3	6·1	5·5
2510	2·00E-03		AAH63812	46·5	46·4	6·1	5·3
2713*	0·0052	1·5E-41	CAA60040	66·6	76·0	6·3	5·4
2714*	0·00042	5·9E-65	AAB34982	74·8	74·0	6·3	5·9
2815		4·5E-41	AAH60518	94·3	90·0	6·2	6·1
3002		5·1E-14	CAA85429	14·1	13·2	6·5	6·1
3607		2·2E-31	P17425	53·5	58·0	6·5	#
3716*	1·00E-06	5·7E-18	AAH88347	74·9	72·7	6·7	5·8
4002		1·5E-05	P02632	13·1	14·3	6·7	7·8
4106		1·4E-07	AAA42020	19·6	20·1	7·0	5·7
4306		5·8E-22	AAC64180	34·0	36·6	6·9	6·2
4404*	8·50E-06	2·9E-19	AAH78930	42·7	45·8	6·9	6·0
4405		2·8E-10	Q91VW4	39·5	40·6	6·9	6·2
4501*	0·043	2·7E-34	AAH89770	48·8	44·1	6·8	5·7
4703*	0·0072	3·5E-23	AAH61765	64·5	59·1	6·8	5·9
4707*	1·60E-05	3·7E-45	CAA24532	69·2	70·7	7·0	6·1
4801*	0·00042		BAB16378	119·9	124·2	6·8	5·5
4809		4·4E-28	AAH89101	100·0	99·7	7·0	5·6
4904*	0·0049	6·9E-53	AAB59717	157·0	165·7	7·0	6·3
5008		1·4E-09	P02692	12·9	14·2	7·3	6·7
5205		6·1E-05	P41034	29·4	31·9	7·2	6·5
5208		3·1E-10	P22789	27·8	33·2	7·4	7·6
5311		8·6E-19	Q920P0	31·0	25·7	7·3	6·8
5314		2·3E-10	Q5BKC8	34·1	37·5	7·4	6·7
5410		7·4E-40	O88655	73·2	74·5	7·3	6·3
5413*	0·0015	2·1E-24	AAH76381	41·7	46·2	7·3	6·7
5602*	1·20E-05	1·1E-46	AAH79381	56·4	56·5	7·1	6·8
5607*	0·003	1·6E-19	AAH81884	52·9	58·1	7·2	6·6
5612		8·6E-22	AAH62069	57·4	61·4	7·4	6·1
5614		5·1E-15	NP036756	55·4	59·0	7·2	6·2
5711*	0·002	2·7E-39	AAH99807	62·9	63·5	7·3	6·2
5801	1·00E-10		AAH85697	87·7	96·2	7·1	6·8
6408*	0·00074	1·1E-25	1RLAB	39·3	34·1	7·7	6·2
6506		8·7E-25	P10760	45·1	43·2	7·6	6·4
6508*	0·0013	4·2E-12	AAH78948	45·5	50·7	7·7	6·7
6709*	1·20E-05	6·1E-40	AAB42378	61·1	59·9	7·7	7·2
7003		1·6E-15	P10111	16·2	18·2	7·9	7·8
7209		4·2E-18	P11348	27·5	25·5	8·1	7·7
7214*	9·70E-06	9·9E-37	P08010	26·6	25·7	8·3	7·3
7304		3·8E-10	AAH87743	34·4	36·1	8·0	8·1
7602		9·8E-33	AAH97369	51·5	57·0	7·9	7·2
7612*	0·00022	9·1E-42	O70199	58·0	55·5	8·2	7·5
7808	0·00067	8·7E-61	A44154	89·5	86·1	8·1	7·9
8312		1·1E-14	AAD42162	30·7	34·5	9·2	8·8
8501		3·5E-19	A23126	49·6	47·1	8·4	6·2

* Indicates identification from Matrix-assisted laser desorption/ionisation−time of flight (MALDI−TOF) spectra and confirmed using liquid chromatography/MS/MS (LCMSMS).
† MALDI and LCMSMS probabilities and the corresponding Entrez code for differentially expressed proteins. The predicted mass/pI is the mass/pI is taken from the Mascot database entry. The mass/pI of the spot is that estimated from the two-dimensional gel.

Discussion

Although numerous hypotheses have been put forward to explain the importance of folic acid in reproduction, the mechanism that underlies its action remains unclear. The present study shows that there are widespread changes in both the metabolism and function of the maternal liver when the diet of pregnant rats is deficient in folic acid and the related methyl donors, methionine and choline. These findings suggest a putative mechanism related to changes in the maternal liver and lipid metabolism in particular.

It is apparent from the differentially abundant proteins involved in metabolic processes (Table 3) that proteins associated with folate metabolism and the methionine cycle change in response to the deficient diets. Two of the major folate binding proteins, 10-formyltetrahydrofolate dehydrogenase

Table 3. Differentially abundant proteins involved in metabolic processes* (Data are mean pixel density with their standard errors of the mean for six rats per group)

Protein index number	Protein name	Function	Control		−F		−FLMLC		
			Mean	SEM	Mean	SEM	Mean	SEM	Fpr
4501	Methionine adenosyltransferase I, α	met cycle	841[a]	231	3099[b]	454	2340[ab]	791	0·035
4809	Formyltetrahydrofolate dehydrogenase	met cycle	252[a]	42	126[b]	29	163[ab]	32	0·080
5801	Dimethylglycine dehydrogenase	met cycle	569[a]	33	354[b]	51	254[b]	40	<0·001
6506	Adenosylhomocysteinase	met cycle	1144[ab]	366	1715[a]	226	833[b]	92	0·074
3716	Glycyl-tRNA synthetase	protein/aa metab	352[a]	24	348[a]	31	702[b]	49	<0·001
4703	Seryl-aminoacyl-tRNA synthetase 1	protein/aa metab	267[a]	26	371[a]	47	541[b]	37	<0·001
3002	DOPDD-dopachrome tautomerase	tyrosine metab	284	17	347·1	75	374	28	0·410
5410	4-hydroxyphenylpyruvate dioxygenase	tyrosine metab	2484[a]	183	3866[b]	591	2272[a]	421	0·040
5413	Fumarylacetoacetase	tyrosine metab	365[ab]	102	561[b]	63	315[a]	53	0·097
6508	Homogentisate 1, 2-dioxygenase	tyrosine metab	791[a]	73	753[a]	34	477[b]	64	0·007
7209	Dihydropteridine reductase	tyrosine metab	1171	260	2450	672	2253	307	0·132
2510	Translation initiation factor eIF-4A I	protein metab	671[a]	157	1205[b]	177	1343[b]	145	0·032
4404	Amino acylase -1	protein metab	453[a]	11	698[b]	67	603[b]	46	0·008
4904	Carbamoyl-phosphate synthase precursor	protein metab	299[a]	38	197[ab]	54	158[b]	36	0·109
5602	Leucine aminopeptidase 3	protein metab	364[a]	34	493[b]	34	442[ab]	43	0·100
6408	Arginase, chain A	protein metab	700[ab]	118	963[a]	191	430[b]	70	0·067
2407	Fructose-1,6-bisphosphatase	energy metab	1339[a]	175	1875[ab]	206	2235[b]	232	0·035
4306	Cytosolic malate dehydrogenase·	energy metab	2550[a]	212	4000[b]	291	3591[b]	226	0·002
5311	Diacetyl/L-xylulose reductase	energy metab	239[a]	34	442[b]	78	296[ab]	70	0·100
5614	Pyruvate kinase	energy metab	263[a]	93	332[a]	73	639[b]	65	0·010
7304	Glyceraldehyde-3-phosphate dehydrogenase	energy metab	1271[a]	134	823[b]	74	1310[a]	101	0·014
5711	Phosphoglucomutase (Pgm1)	energy metab	1044[ab]	65	1203[a]	28	998[b]	67	0·064
7602	UDP-glucose pyrophosphorylase	energy metab	422[a]	49	957[b]	73	745[c]	57	<0·001
7808	Aconitate hydratase, mitochondrial precursor	energy metab	212[ab]	66	153[a]	26	303[b]	36	0·110
8501	Phosphopyruvate hydratase (enolase)	energy metab	565[a]	116	196[b]	51	215[b]	49	0·007
3607	Hydroxymethylgluyaryl - CoA synthase-cytoplasmic	lipid metab	210[a]	19	50[b]	33	103[b]	34	0·005
4002	Fatty acid binding protein	lipid metab	649[a]	69	980[b]	94	960[b]	55	0·011
5008	Fatty acid binding protein	lipid metab	5042[a]	239	5480[a]	207	4081[b]	444	0·020
5612	Dihydrolipoamide dehydrogenase	lipid metab	233[a]	10	414[b]	72	341[ab]	75	0·134
8312	3-hydroxyacyl-CoA dehydrogenase precursor	lipid metab	2789[ab]	586	3616[a]	461	1742[b]	259	0·034

Data analysed by one-way ANOVA (Fpr) followed by Fischer's unprotected test. Mean values within a row with unlike superscript letters are significantly different ($P<0.05$).
−F, diet deficient in folic acid; −FLMLC, diet deficient in all three key methyl donors, folic acid, choline and methionine.
* For details of diets and procedures, see Methods.

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Table 4. Differentially abundant structural proteins classified by function (Data are mean pixel density with their standard errors of the mean for six rats per group)

Protein index number	Protein name	Function	Control Mean	SEM	−F Mean	SEM	−FLMLC Mean	SEM	Fpr
606	Calreticulin precursor	ER	551[a]	83	272[b]	58	206[b]	37	0·006
1306	Apolipoprotein E precursor	ER	738[ab]	91	906[a]	82	625[b]	69	0·099
1308	Regucalcin	ER	3418[ab]	321	4600[a]	664	2145[b]	158	0·005
1509	Tubulin, β, 2	ER	230	30	441	157	388	40	0·303
2713	Annexin VI (67Kda)	ER	205[a]	17	263[ab]	27	298[b]	26	0·054
2714	dnaK-type molecular chaperone grp75 precursor	ER	1582[a]	115	1604[a]	184	2291[b]	232	0·032
2815	Transitional endoplasmic reticulum ATPase (EC 3·6·1·-)	ER	284[a]	58	121[b]	14	153[b]	26	0·018
5604	Aldehyde dehydrogenase family 7, member A1	ER	460[a]	85	929[b]	107	574[ab]	132	0·022
5607	Aldehyde dehydrogenase 1 family, member B1	ER	942[a]	116	1460[ab]	129	1495[b]	245	0·081
7003	Peptidylprolyl isomerase	ER	380[a]	87	137[b]	48	171[ab]	39	0·028
7612	UDP-glucose 6-dehydrogenase	ER	235[a]	36	279[a]	45	471[b]	34	0·002
4801	Vinculin (Metavinculin)·	bile production	117[a]	14	65[b]	12	96[ab]	16	0·081
5208	Hydroxysteroid sulfotransferase	bile production	810[a]	82	800[a]	104	420[b]	45	0·005
5314	3-alpha-hydroxysteroid dehydrogenase	bile production	609[a]	79	555[ab]	50	391[b]	33	0·042
1114	Thiol Specific antioxidant	Oxidative stress	843[a]	63	1047[ab]	115	1116[b]	33	0·065
2203	Gamma-glutamylcysteine synthetase light chain	Oxidative stress	243[a]	20	273[ab]	56	390[b]	58	0·037
4106	Retinol binding protein (serum precursor)	Oxidative stress	240	23	214	77	344	22	0·168
5205	α-tocopherol transfer protein (II)	Oxidative stress	833[a]	53	1072[b]	82	734[a]	72	0·011
6709	Catalase (EC 1·11·1·6)	Oxidative stress	971[a]	79	552[b]	101	822[ab]	106	0·032
7214	Glutathione S-transferase Mu 2 (GSTM2-2)	Oxidative stress	1667[a]	338	2285[ab]	619	3329[b]	417	0·085
206	14-3-3 protein gamma-subtype ((Protein kinase C inhibitor protein 1)	Other	490[a]	23	790[ab]	145	931[b]	135	0·058
2005	Cellular retinal binding protein	Other	280	56[a]	105[b]	11	167[ab]	31	0·015
2401	Alpha-1-macroglobulin	Other	116	19[a]	195[b]	23	126[a]	26	0·056
4405	Uroporphyrinogen decarboxylase	Other	103	5[a]	132·7[b]	12	153[b]	9	0·007
4707	Serum albumin precursor	Other	3393	1151	1887	100	2430	162	0·308

Data analysed by one-way ANOVA (Fpr) followed by Fischer's unprotected test. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).

−F, diet deficient in folic acid; −FLMLC, diet deficient in all three key methyl donors, folic acid, choline and methionine; ER, endoplasmic reticulum.
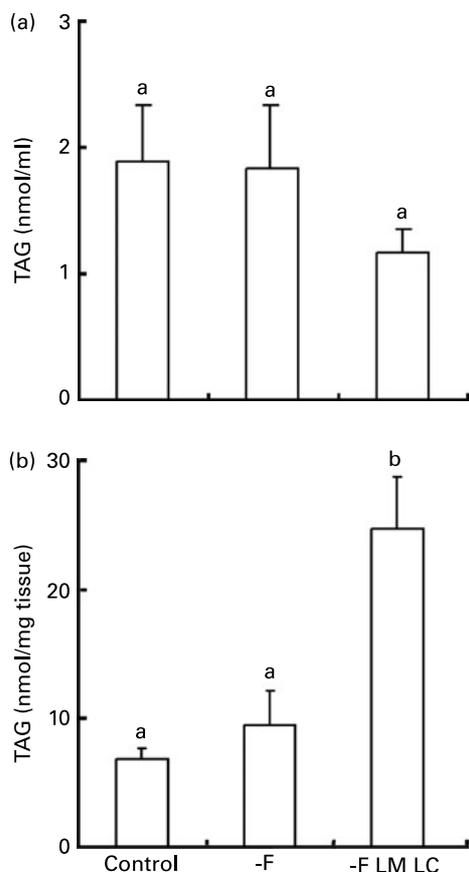
* For details of diets and procedures, see Methods.

Folate deficiency and the maternal liver

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Fig. 1. TAG concentrations in maternal serum (a) and maternal liver (b). Values are means with their standard errors of the mean. Control *n* 8; diet deficient in folic acid (−F) *n* 6; diet deficient in all three key methyl donors, folic acid, choline and methionine (−FLMLC) *n* 7. Data analysed by one-way ANOVA followed by Fischer's unprotected test. Columns with unlike superscript letters are significantly different ($P < 0.05$). For details of diets and procedures, see Methods.

and dimethyl glycine dehydrogenase are lower in both the −F and −FLMLC diet groups (Table 3), a result that is consistent with the reduced hepatic folate stores in these animals[9]. There are also differences in the abundance of proteins associated

with the methionine cycle in both groups. There is an increase in methionine adenosyl transferase and a tendency for an increase in adenosyl homocysteinease in the animals fed the −F diet, suggesting that methionine and choline compensate for the reduced availability of folates when they are available. Glycine and serine are important donors of methyl groups, which enter the methionine cycle *via* derivatives of folic acid. Plasma concentrations of both amino acids increase especially in the animals fed −FLMLC diets[9] and the increase in the abundance of the glycyl- and seryl-t-RNA synthetases is probably a response to these changes.

Although there is no direct involvement of folic acid or its derivatives in tyrosine metabolism, a number of differentially abundant proteins are involved in the metabolism of this amino acid. Plasma tyrosine concentrations increase relative to the complete diet by 45 % in animals fed the −F diet and decrease by 12 % in animals fed the −FLMLC diets[9], suggesting that these changes are having differential effects on tyrosine metabolism. Tyrosine is produced from phenylalanine *via* the enzyme phenylalanine hydroxylase, which requires tetrahydrobiopterin as an essential cofactor in the reaction. There are several reports of a link between folate and biopterin metabolism, possibly related to the structural and functional similarities of the folate and biopterin coenzymes[13]. The present results add further circumstantial evidence to suggest that folate deficiency has an effect on tetrahydrobiopterin metabolism. As tetrahydrobiopterin is thought to be required for successful neural tube closure[14], this may be a possible mechanism for an indirect effect of folate deficiency on fetal development.

Beyond the changes directly associated with the metabolism of folates and the methionine cycle, the relative abundance of proteins associated with hepatic protein, glucose and lipid metabolism is also influenced by diets deficient in folic acid and the related methyl donors (Table 3). The changes in protein metabolism appear to be rather non-specific and probably reflect differences in growth as the livers of animals fed the −FLMLC diet are some 22 % smaller than those of animals fed the complete diet. This suggests that there is a change in protein turnover that may be related to a limitation in sulphur amino acids in the animals fed the −FLMLC diet. In contrast,

Table 5. Relative mRNA levels in maternal liver*

(Values are means with their standard errors of the mean for six rats per group)

Diet...	Control		−F		−FLMLC		
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Fpr
Acc-1	17·38[a]	0·72	12·80[b]	1·24	13·00[b]	0·97	0·009
L-CPT-1	7·69[a]	0·44	9·02[a]	1·80	12·45[b]	1·43	0·037
C/EBP-α	20·64	2·00	25·85	3·89	25·46	1·35	0·328
PPAR-α	0·99[a,b]	0·06	0·89[a]	0·10	1·26[b]	0·08	0·013
PPAR-γ	0·29[a]	0·04	0·34[ab]	0·04	0·46[b]	0·04	0·031
SREBP-1c	1·02[a]	0·18	0·51[b]	0·15	0·57[b]	0·08	0·049
Gadd153	34·98	2·70	31·60	2·03	36·22	4·08	0·556
P57	23·37	1·39	23·52	2·34	22·19	1·67	0·857

All data are expressed as relative expression corrected for 18S rRNA. Data analysed by one-way ANOVA (Fpr) followed by Fischer's unprotected test. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).

−F, diet deficient in folic acid; −FLMLC, diet deficient in all three key methyl donors, folic acid, choline and methionine; Acc, acetyl CoA carboxylase; L-CPT-1, carnitine palmitoyl transferase; C/EBP-α, CCAAT enhancer binding protein α; SREBP-1c, sterol response element-binding protein 1c.

* For details of diets and procedures, see Methods.

the changes in carbohydrate and lipid metabolism suggest more specific alterations, albeit to a diverse range of processes including gluconeogenesis, glycogen synthesis, cholesterol metabolism, the malate-aspartate shuttle and mitochondrial β-oxidation. In general, the changes are greater and more widespread in the animals fed the −FLMLC diet, suggesting that the multiple deficiency is exacerbating the impact on energy and lipid metabolism. However, it is difficult to make specific predictions on the metabolic effects of these changes, since, in addition to transcriptional control, the activities of several of these enzymes are tightly regulated by a combination of allosteric effectors and phosphorylation.

The increase in hepatic TAG that occurs in the animals fed the −FLMLC diets is clearly associated with the changes in energy metabolism. Excessive synthesis and reduced oxidation of fat appear to cause steatosis in both alcoholic liver injury and non-alcoholic fatty liver disease[15]. However, there is no evidence for this in the case of folate deficiency. The hepatic mRNA for the key enzyme regulating fatty acid synthesis (acetyl CoA carboxylase-1) is reduced in animals from both the −F and −FLMLC groups. At the same time, the mRNA for carnitine palmitoyl transferase-1 (Table 5) the key regulator of lipid oxidation is increased in the livers of animals fed −FLMLC diets but not in those fed the −F diet. As the mRNA levels reflect the enzyme activities[16], these findings suggest a reduction in the synthesis and an increase in the oxidation of fatty acids, opposing the accumulation of TAG in the −FLMLC group. It appears that the responses to the −F and −FLMLC diets differ because the mRNA for PPAR-α and PPAR-γ, which regulate fatty acid oxidation, are only increased in the animals fed the −FLMLC diets, whereas SREBP-1c, an important regulator of hepatic lipogenesis and cholesterol synthesis[17], is decreased by both −F and −FLMLC diets. The PPAR and SREBP-1c also regulate other key metabolic enzymes and the differential abundance of several other proteins is probably due to these transcription factors. For example the abundance of pyruvate kinase, which is regulated by PPAR-α[18], is also increased in animals fed the −FLMLC diet.

PPAR-α has also been implicated in other models of hepatic steatosis, such as alcoholic fatty liver[19] or diets deficient in methionine and choline[20]. The steatosis caused by folate deficiency during pregnancy appears to differ from other forms of steatosis, in that there is no increase in markers of liver damage such as elevated serum aspartate aminotransferase and alanine aminotransferase or an increase in the expression of gadd153, p57 and CCAAT enhancer binding protein-α. This suggests that although there is an increase in the hepatic TAG content, it is not sufficient to induce cell damage. The metabolic changes initiated by activation of SREBP and PPAR appear to be sufficient to minimise the accumulation of lipid and this may in turn prevent the damage to cells that is responsible for initiating the pathological changes seen in models of alcoholic liver injury[21].

Since production and oxidation of fatty acids does not account for the accumulation of TAG, the hepatic steatosis in animals fed the −FLMLC diet is probably caused by a reduced rate of TAG export. Evidence for this comes from the differential abundance of a number of proteins associated with the secretion of LDL, including fatty acid binding proteins, calreticulin and the apoB precursor. All of these proteins are associated with intracellular lipid handling and export via the endoplasmic reticulum and Golgi apparatus. There is also a change in the abundance of the transitional endoplasmic reticulum ATPase (down regulated in both −F and −FLMLC), which transports vesicles within the cell via the cytoskeleton[22]. All of these changes would be consistent with a reduced rate of TAG export leading to steatosis in the animals fed the −FLMLC diet. Furthermore, the steatosis caused by methyl-deficient diets is similar to that found in animals with a deletion of the PEMT gene[23]. As phosphatidyl choline produced by PEMT is used for the production of lipoproteins, it is a strong candidate for the underlying target of folate deficiency. The PEMT enzyme is localised in a subfraction of endoplasmic reticulum membranes, known as mitochondria-associated membranes[24], where it is a major user of S-adenosyl methionine[23,25]. The change in the abundance of the dnaK type molecular chaperone glucose regulated protein 75 precursor is evidence for a change in these membranes, as it has been suggested that this protein is associated with mitochondrial import, antioxidant defence and lipid metabolism[26].

In addition to lipoprotein production, significant amounts of the phosphocholine produced de novo by PEMT are used for the production and secretion of phosphatidic acid, lyso-phosphatidyl choline and taurocholic acid found in bile[27]. The down regulation of vinculin, a cytoskeletal protein specifically associated with bile canniculus[28], and two proteins associated with bile production, 3-α hydroxysteroid dehydrogenase and hydroxysteroid sulphotransferase[29], suggest that bile production is reduced in animals fed the −F and −FLMLC diets. Reduced bile production or secretion may have widespread effects, particularly on the digestion of lipids and the bile acid cycle. Interestingly, in addition to the production of bile acids, 3-α hydroxysteroid dehydrogenase and hydroxysteroid sulphotransferase also have a critical role in the clearance of steroid hormones by the liver[30–32]. It is therefore possible that folate deficiency may have an indirect effect on systemic steroid hormone levels as a result of a reduced clearance rate.

Comparison of the soluble hepatic proteome of the pregnant female with the male rat exposed to folate-deficient diets[33] shows a number of common features. These include 3-α hydroxysteroid dehydrogenase, endoplasmic reticulum-associated proteins (glucose regulated protein 75) as well as changes in the antioxidant capacity. Previous studies have shown that oxidative stress is increased in folate-deficient animals and is strongly correlated to the accompanying elevation in plasma homocysteine[33,34]. The up regulation of γ-glutamylcysteine synthetase and glutathione S-transferase is greater in the livers of animals fed the −FLMLC diet despite the fact that plasma homocysteine concentrations are similar in animals fed −F and −FLMLC diets[9]. Since both groups are exposed to similar levels of homocysteine, these results suggest that the response to oxidative damage is more likely to be induced by the development of steatosis rather than a direct effect of elevated homocysteine. A similar observation of increased lipid peroxidation has been made in animals that develop a fatty liver due to a defect in VLDL secretion[35]. In both cases, it appears that the fatty liver due to a defect in TAG export is associated with increased oxidative stress caused by the accumulation of TAG.

The present study has shown that changes in the flux through the methionine cycle cause widespread changes in lipid metabolism and export from the maternal liver, suggesting that lipid metabolism may be a common factor linking folate status, BMI and NTD occurrence. During pregnancy, fatty acids derived from lipoprotein-associated TAG provide an important source of lipid for the developing fetus[36]. By interfering with the export of lipoprotein, folate deficiency may disrupt this supply. It is striking that NTD and other developmental anomalies are more common in infants born to obese women[37]. A BMI > 30 doubles the risk of having a child with a NTD compared with normal-weight women and, critically, this increased risk does not appear to be modified by folic acid supplementation[38]. Obese individuals frequently have steatotic livers, suggesting a possible link between hepatic lipid metabolism and NTD. The close interrelationship between folates, the methionine cycle and fat metabolism suggest that high-fat, low-folate diets, typical of those found in Western Europe, may be delivering a double blow to the delivery of essential lipids to the developing fetus.

Acknowledgements

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