Maternal hypothyroidism in the rat influences placental and liver glycogen stores: fetal growth retardation near term is unrelated to maternal and placental glucose metabolic compromise

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Abstract

Maternal hypothyroidism impairs fetal growth in the rat, but the mechanisms by which this occurs are unknown. Since the fetus derives its glucose supply from the mother, and maternal thyroidectomy may disturb maternal and placental glucose metabolism, we postulated that maternal and/or placental glucose metabolic compromise may contribute to fetal growth retardation in hypothyroid dams. Feto-placental growth, tissue glycogen stores and glucose levels in sera and amniotic fluid were determined in rat dams partially thyroidectomized (TX) before pregnancy and in euthyroid controls. Fetal body weight at 16, 19 and 21 days gestation (d.g.) was related to pre-mating maternal serum total thyroxine (TT₄) levels; permanent fetal growth retardation occurred in severely (TX_s; pre-mating maternal serum TT₄ ≤ 16.19 nM) – but not in moderately (TX_m) – hypothyroid dams. In TX_s dams, glycogen

concentration was elevated in maternal liver and in the fetal side of the placenta at 16 and 19 d.g., and in the maternal side of the placenta at 19 and 21 d.g., despite maternal euglycemia. In contrast, fetal liver glycogen concentration was deficient in TX_m dams at 19 d.g. and in TX_s dams at 19 and 21 d.g., and fetal hypoglycemia occurred in TX_s dams at 21 d.g. Multiple regression analyses indicate that these fetal deficits are strongly associated with the retardation in fetal growth, while the elevated maternal liver and placental glycogen concentrations have no impact on fetal growth near term. The mechanisms by which severe maternal hypothyroidism permanently retards rat fetal growth remain to be determined.

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Introduction

Maternal hypothyroidism influences feto-placental growth in rats, though the outcome depends on the timing of thyroidectomy in relation to conception and the degree of hypothyroidism. Thyroidectomy of rats before pregnancy has no effect on placental growth, but retards fetal growth, either transiently in moderately hypothyroid dams (Pickard *et al.* 1999) or permanently in severely hypothyroid dams (Morreale de Escobar *et al.* 1985, Leonard *et al.* 1999). In contrast, induction of moderate or severe maternal hypothyroidism soon after conception permanently retards placental and fetal growth (Porterfield *et al.* 1975, Bonet & Herrera 1988, Hendrich & Porterfield 1992).

The mechanisms by which maternal thyroid status regulates fetal development are poorly understood. Direct action is possible, since materno-fetal thyroxine (T₄) transfer occurs in rat pregnancy, and fetal tissues express 5'-deiodinases and nuclear 3,5,3'-tri-iodothyronine (T₃)

receptors (TR) (Porterfield & Hendrich 1993, Pickard *et al.* 1997, Morreale de Escobar *et al.* 2000). The placenta also accumulates and metabolizes maternal T₄ and T₃ (Calvo *et al.* 1992) and expresses TR (Leonard *et al.* 2001); thus maternal thyroid hormone may regulate fetal development via effects on the placenta. Finally, other indirect mechanisms involving maternal tissues (Bonet & Herrera 1988, Hendrich & Porterfield 1992) may be influential.

In dams made hypothyroid soon after conception, maternal metabolic compromise occurs (Bonet & Herrera 1988) which, together with reduced placental size, may restrict the provision of nutrients to the fetus, resulting in permanent fetal growth retardation. Whether dams made hypothyroid before pregnancy also suffer metabolic compromise is unknown. Although placental size is normal in the latter model, moderate maternal hypothyroidism compromises placental expression of glucose transporter (GLUT) protein isoforms (Pickard *et al.* 1999). Furthermore, T₃ administration to rat dams during late pregnancy depletes placental glycogen stores (Shafir *et al.*

1994). Thus maternal thyroid status may regulate placental glycogen homeostasis and glucose transport and hence the supply of maternal glucose to the fetus. Since glucose serves as the primary fetal growth substrate, and the rat fetus is incapable of gluconeogenesis (Jones & Rolph 1985, Girard *et al.* 1992), we postulate that in dams made hypothyroid before pregnancy, permanent fetal growth retardation occurs as a consequence of disturbances in maternal and placental glycogen storage and hence glucose supply to the fetus (i.e. maternal and/or placental glucose metabolic compromise).

To test this hypothesis, we first examined the relationship between pre-mating maternal thyroid status and fetal body weight in partially thyroidectomized (TX group) and euthyroid rat dam pregnancies, and exploited the relationship near term to assign TX dam pregnancies at all stages of gestation to severely hypothyroid (TX_s; permanently growth-retarded) and more moderately hypothyroid (TX_m) groups. We then examined whether moderate and severe maternal hypothyroidism influences placental, maternal liver and fetal liver glycogen stores, as well as maternal and fetal glycemic status. Finally, we addressed whether compromise in maternal liver and placental glycogen concentrations contributes to permanent fetal growth retardation in TX dams.

Materials and Methods

Materials

All enzymes and fine chemicals were from Sigma-Aldrich Company Limited (Gillingham, Dorset, UK). The total T₄ (TT₄) RIA kit was from NETRIA (London, UK) and the total T₃ (TT₃) RIA kit from BM Browne (UK) Limited (Reading, Berks, UK).

Animal model

Sprague-Dawley female rats (proven breeders) were partially thyroidectomized by surgical removal of the lower one-half to two-thirds of each lobe, sparing the parathyroids, and allowed to recover for at least 2 weeks. Circulating TT₄ levels were determined and the animals mated overnight with normal males; the morning of appearance of a vaginal plug was designated day 0 of pregnancy. Normal (N group) females served as the control group. Animals were maintained in the local animal house facilities at 22 °C on a cycle of 14 h light : 10 h darkness, and allowed free access to a standard small laboratory animal diet and water. The water of the TX group was supplemented with 0.1% (w/v) calcium lactate throughout.

Animals were stunned and killed by cervical dislocation at 16, 19 and 21 days gestation (d.g.); fetal thyroid hormone secretion occurs from 17.5 d.g. (Morreale de

Escobar *et al.* 1985) and parturition at 22 d.g. Maternal blood was collected by cardiac puncture and the uterine horns were removed to ice. Fetuses and placentae were isolated and weighed, then fetal tissues were dissected and weighed. Tissue pools (from ≥ three fetuses) were immediately frozen on dry ice and stored at −20 °C until assay.

Samples were collected from 17 different batches of animals. In the light of initial findings, the tissue collection protocol was modified for the latter seven batches to include maternal liver (left lateral lobe), amniotic fluid and fetal blood; placental discs were also separated into maternal (junctional zone) and fetal (labyrinth) sides.

All experimental procedures complied with the Animal (Scientific Procedures) Act 1986 of the United Kingdom.

Glycogen and glucose determination

Liver and placental samples were deproteinized by homogenization with 5 volumes ice-cold 0.6 M perchloric acid, and glycogen was determined by a standard procedure, employing fungal glucoamylase for hydrolysis (Keppler & Decker 1984). A rabbit liver glycogen preparation (0.45 M) was included with each batch of samples to verify complete hydrolysis.

Glucose was determined in neutralized tissue homogenates and hydrolysates, sera and amniotic fluid using a standard spectrophotometric procedure with hexokinase and glucose 6-phosphate dehydrogenase (Keppler & Decker 1984); glucose standards (10–250 nmol) were included in each assay. Glycogen concentrations, expressed as μmol glucose/g tissue wet weight, were corrected for endogenous glucose (Keppler & Decker 1984).

Thyroid hormone determination

Levels of TT₄ and TT₃ were determined in maternal serum by RIA. Inter- and intra-assay coefficients of variance were, respectively, 5.8 and 4.5% for TT₄, and 5.7 and 3.3% for TT₃.

Statistical analysis

Data from N and TX dam pregnancies were compared by one-way ANOVA; Fisher's protected least significance differences (PLSD) were determined for *post hoc* analysis. Homogeneity of variance for all groups was assessed using Bartlett's test for samples of unequal size and, where necessary, a square root or log_e transformation was applied before analysis. For simple and multiple regression analyses, linearity of the data was determined by a runs test. Statistical analyses were performed using Statview 1.03 (Abacus Concepts, Inc., Berkeley, CA, USA) and

Figure 1 Relationship between fetal body weight near term and pre-mating maternal serum TT_4 level. Data derived from N ($\circ$; $n=20$) and TX ($\bullet$; $n=34$) dam pregnancies at 21 d.g. were consistent with a hyperbolic relationship (left panel). Regression of fetal body weight on the reciprocal of pre-mating maternal serum TT_4 (right panel) yielded a linear relationship ($y=-9.042x+5.088$; $r=-0.547$; $P<0.001$). The 99% confidence interval of the intercept at 5.09 g was calculated, and the grey line indicates extrapolation of the lower limit of 4.73 g across to the upper 99% confidence limit of the regression line. This corresponds to a pre-mating maternal serum TT_4 level of 16.19 nM, at or below which permanent fetal growth retardation is considered to occur.

GraphPad Prism 2.0c (GraphPad Software, Inc., San Diego, CA, USA); statistical significance was defined as $P<0.05$. Results are expressed as means $\pm$ S.E.M.

Results

Relationship between fetal body weight and pre-mating maternal serum TT_4 levels

Previous studies with the TX dam model have shown that in moderate maternal hypothyroidism, fetal body weight is reduced at 16 d.g. but normal at 21 d.g. (Pickard *et al.* 1999), whereas in severe maternal hypothyroidism, late fetal growth is also retarded (Leonard *et al.* 1999). Indeed in the present study, fetal body weights at 16–21 d.g. were found to vary with pre-mating maternal serum TT_4 levels, in a manner consistent with a hyperbolic relationship at each age (Fig. 1 left panel and data not shown). Consequently, fetal body weight exhibited an inverse linear relationship with the reciprocal of pre-mating maternal serum TT_4 at 16 d.g. ($r=-0.526$; $P<0.001$; $n=61$), 19 d.g. ($r=-0.339$; $P=0.011$; $n=55$) and 21 d.g. (Fig. 1 right panel). The relationship at 21 d.g. allowed statistical determination of the pre-mating maternal serum TT_4 level which would be expected to give rise to permanently growth-retarded fetuses. The 99% confidence interval of the y -axis intercept in the reciprocal plot was calculated (Altman & Gardner 1989), and the lower limit extrapolated to the upper 99% confidence limit of the regression line, corresponding to a pre-mating TT_4 level of 16.19 nM (Fig. 1 right panel). This value was used to assign TX dams at all stages of gestation to moderately (TX_m dams; pre-mating $TT_4 \leq 16.19$ nM) and severely

(TX_s dams; pre-mating $TT_4 = 16.19$ nM) hypothyroid groups, in order to better explore how severe maternal hypothyroidism retards late fetal growth.

Maternal thyroid status

Pre-mating maternal serum TT_4 levels were reduced in TX_m and TX_s dams compared with N dams, and in TX_s dams compared with TX_m dams (Table 1), as expected. Within each treatment group, pre-mating TT_4 levels were similar irrespective of the stage of gestation studied.

Maternal serum TT_4 levels were reduced in both TX dam groups relative to N dams at all ages during pregnancy, and in TX_s dams relative to TX_m dams at 16 and 19 d.g. (Table 1). Maternal serum TT_4 levels at each stage

Table 1 Serum thyroid hormone levels in N, TX_m and TX_s dams before mating and during pregnancy

	Dam	Pre-mating	Pregnancy	
		TT_4 (nM)	TT_4 (nM)	TT_3 (nM)
16 d.g.	N	53.5 ± 2.1	40.9 ± 1.9	1.05 ± 0.12
	TX _m	19.9 ± 0.9^c	17.8 ± 1.5^c	0.57 ± 0.10^c
	TX _s	$11.4 \pm 0.5^{c,f}$	$12.2 \pm 0.6^{c,e}$	0.41 ± 0.07^c
19 d.g.	N	55.9 ± 3.4	27.8 ± 1.2	1.03 ± 0.12
	TX _m	20.4 ± 0.9^c	13.2 ± 1.2^c	0.59 ± 0.08^a
	TX _s	$10.4 \pm 0.5^{c,f}$	$9.9 \pm 1.0^{c,d}$	0.34 ± 0.06^c
21 d.g.	N	56.5 ± 3.1	19.8 ± 1.6	0.69 ± 0.09
	TX _m	19.7 ± 0.8^c	10.5 ± 1.3^c	0.52 ± 0.14
	TX _s	$11.2 \pm 0.7^{c,f}$	8.3 ± 1.0^c	0.45 ± 0.15^b

^a $P<0.02$, ^b $P<0.01$ and ^c $P<0.001$ TX_m or TX_s vs N dam; ^d $P<0.05$, ^e $P<0.005$ and ^f $P<0.001$ TX_s vs TX_m dam; $n \geq 13$ different pregnancies.

Table 2 Litter size and feto-placental growth in N, TX_m and TX_s dam pregnancies

	Dam	Litter size	Tissue weight (mg)					
			Fetal body	Fetal brain	Fetal liver	Placenta		
						Whole	Maternal	Fetal
16 d.g.	N	16.7 ± 0.6	489 ± 7	53.4 ± 0.8	28.6 ± 0.8	326 ± 9	153 ± 5	147 ± 4
	TX _m	12.6 ± 1.0 ^d	449 ± 8 ^b	49.5 ± 1.2 ^a	27.1 ± 1.7	336 ± 14	168 ± 7	146 ± 8
	TX _s	12.4 ± 0.7 ^d	439 ± 6 ^d	48.7 ± 1.2 ^c	24.4 ± 0.9 ^d	334 ± 14	153 ± 10	136 ± 8
19 d.g.	N	15.9 ± 0.8	2240 ± 32	130.0 ± 1.4	161.5 ± 3.1	526 ± 18	164 ± 10	292 ± 9
	TX _m	12.6 ± 0.6 ^b	2143 ± 41	129.7 ± 2.8	155.1 ± 6.0	519 ± 19	153 ± 10	296 ± 13
	TX _s	11.1 ± 0.6 ^d	2034 ± 39 ^d	125.7 ± 1.1	144.4 ± 3.1 ^a	546 ± 11	167 ± 8	297 ± 15
21 d.g.	N	16.8 ± 0.8	4936 ± 110	191.0 ± 2.3	290.5 ± 8.8	570 ± 22	152 ± 11	340 ± 12
	TX _m	12.3 ± 1.0 ^d	4697 ± 154	185.5 ± 3.2	243.0 ± 11.3 ^c	589 ± 23	147 ± 18	304 ± 8
	TX _s	10.1 ± 0.6 ^d	4163 ± 134 ^{d, #}	179.4 ± 2.7 ^d	220.5 ± 8.5 ^d	578 ± 15	149 ± 12	318 ± 9

^a*P*<0.02, ^b*P*<0.005, ^c*P*<0.002 and ^d*P*<0.001 TX_m or TX_s vs N dam; [#]*P*<0.001 TX_s vs TX_m dam; *n* ≥ 6 different pregnancies for weights of maternal and fetal placenta and ≥ 12 for all other parameters.

of pregnancy were highly correlated with pre-mating serum TT₄ levels (*r*=0.658; *P*<0.001; *n*=53).

Compared with N dams, maternal serum TT₃ levels were lower in TX_m and TX_s dams at 16 and 19 d.g. (Table 1). Levels declined between 19 and 21 d.g. in N dams (*P*<0.05), but remained stable in TX_m and TX_s dams, so that at 21 d.g., only TX_s dams showed depressed serum TT₃ levels relative to N dams.

Litter size and feto-placental growth

The number of fetuses carried by TX_m and TX_s dams was reduced at all ages relative to N dams (Table 2). Litter size in TX_s dams was lower (*P*<0.05) at 21 d.g. than at 16 d.g., and regression analysis confirmed a linear relationship (*r*=−0.304; *P*=0.014; *n*=65) between litter size and gestational age over the period 16–21 d.g. in TX_s dams alone.

Fetal body weight at 21 d.g. was reduced (84% control value) in TX_s dams only (Table 2), as expected, and less marked fetal body weight deficits (c. 90% control value) were also seen in TX_s dams at 16 and 19 d.g. Fetuses from TX_m dams, in contrast, exhibited reduced fetal body weight at 16 d.g. only (Table 2). Fetal brain weight was reduced by a similar extent to fetal body weight in TX_m and TX_s dams at 16 d.g., but normalized in both groups by 19 d.g. (Table 2). It was however again reduced in TX_s dams at 21 d.g. (Table 2). Fetal brain:body weight ratios were elevated in TX_s dams at 19 d.g. (0.062 ± 0.001 vs 0.058 ± 0.001 for controls; *P*<0.05; *n*=22) and at 21 d.g. (0.044 ± 0.001 vs 0.039 ± 0.001 for controls; *P*<0.05; *n*=20). Fetal liver weights were deficient in TX_s dams at all ages and in TX_m dams at 21 d.g. (Table 2). Fetal liver:body weight ratios were reduced at 21 d.g. in TX_m dams (0.052 ± 0.001; *P*<0.05; *n*=12) and TX_s dams (0.052 ± 0.002; *P*<0.05; *n*=20) compared with N dams (0.058 ± 0.002; *n*=23).

In N dams, the weight of the maternal side of the placenta remained constant over the period studied, whereas that of the fetal side more than doubled (Table 2), as expected (Davies & Glasser 1968), so that near term the fetal side accounted for two-thirds of total placental mass, in keeping with published data (Ne'eman *et al.* 1987). The weight of the placenta, either whole or after separation into maternal and fetal sides, was normal in all TX dams (Table 2).

Tissue glycogen levels

For all tissues from euthyroid pregnancies, glycogen concentrations and their ontogeny agreed with published data (Gruppuso & Brautigan 1989, Shafir & Barash 1991).

Fetal liver glycogen concentration and content were low in N dams at 16 d.g., then increased markedly as gestation progressed (Table 3). Relative to N dams, fetal liver glycogen levels were lower in TX_m and TX_s dams at 19 d.g. and in TX_s dams at 21 d.g. Furthermore, upon combining data from all pregnancies, fetal liver glycogen concentration was inversely related to the reciprocal of pre-mating maternal serum TT₄ at both 19 d.g. (*r*=−0.469; *P*=0.014; *n*=27) and 21 d.g. (*r*=−0.522; *P*=0.001; *n*=31). However, fetal liver glycogen concentration was directly related to fetal body weight at 19 d.g. (*r*=0.656; *P*<0.001) and at 21 d.g. (*r*=0.743; *P*<0.001), so that depressed fetal liver glycogen stores in TX dams may occur secondary to retarded fetal growth. Indeed multiple regression analysis of these data revealed that fetal liver glycogen concentration was significantly related to fetal body weight at 19 d.g. (*P*=0.001) and at 21 d.g. (*P*<0.001), but not to the reciprocal of pre-mating maternal serum TT₄ (albeit *P*=0.087 at 19 d.g.).

Maternal liver glycogen concentration, in contrast, declined between 16 and 21 d.g. in N dams (Table 3), and was elevated in TX_s dams at 16 and 19 d.g. relative to

Table 3 Maternal and fetal liver glycogen levels in N, TX_m and TX_s dam pregnancies

	Dam	Fetal liver		Maternal liver
		Concentration (μmol/g)	Content (μmol)	Concentration (μmol/g)
16 d.g.	N	2.1 ± 0.4	0.05 ± 0.01	223.9 ± 15.7
	TX _m	2.4 ± 0.4	0.07 ± 0.01	257.5 ± 21.1
	TX _s	2.2 ± 0.4	0.06 ± 0.01	300.3 ± 20.1 ^c
19 d.g.	N	122.8 ± 12.4	18.80 ± 1.72	113.3 ± 14.7
	TX _m	76.1 ± 11.7 ^b	11.43 ± 1.81 ^a	102.7 ± 27.9
	TX _s	53.9 ± 7.4 ^e	7.90 ± 1.20 ^e	172.1 ± 33.3 ^a
21 d.g.	N	415.8 ± 34.6	118.31 ± 9.99	50.1 ± 13.2
	TX _m	420.7 ± 47.4	116.17 ± 16.60	36.8 ± 16.6
	TX _s	266.2 ± 35.9 ^{b, #}	61.33 ± 9.54 ^{d, ###}	68.6 ± 16.7

^a $P < 0.05$, ^b $P < 0.02$, ^c $P < 0.01$, ^d $P < 0.002$ and ^e $P < 0.001$ TX_m or TX_s vs N dam; [#] $P < 0.05$ and ^{##} $P < 0.01$ TX_s vs TX_m dam; $n \geq 8$ different pregnancies for fetal liver, and ≥ 4 for maternal liver.

controls. Regression of maternal liver glycogen concentration on the reciprocal of pre-mating maternal serum TT₄ yielded significant linear relationships at 16 d.g. ($r = 0.455$; $P = 0.026$; $n = 24$) and at 19 d.g. ($r = 0.533$; $P = 0.011$; $n = 22$). However, reductions in litter size and fetal body weight may have contributed to elevated maternal liver glycogen levels in TX_s dams by placing less demand on maternal glucose reserves. In this regard, maternal liver glycogen concentration in N, TX_m and TX_s dams combined was negatively correlated with litter size at 16 d.g. ($r = -0.459$; $P = 0.02$; $n = 24$) and at 19 d.g. ($r = -0.568$; $P = 0.006$; $n = 22$), but was unrelated to fetal body weight. Multiple regression analysis of combined 16 and 19 d.g. data was therefore performed; this yielded a significant relationship ($R = 0.813$; $P < 0.001$; $n = 46$), maternal liver glycogen concentration being directly related to the reciprocal of pre-mating maternal serum TT₄ ($P = 0.039$) and inversely related to the number of fetuses ($P = 0.018$) and days gestation ($P < 0.001$). Thus, elevated maternal liver glycogen concentration in TX_s dams at 16 and 19 d.g. may occur due to reductions in litter size and circulating maternal thyroid hormone levels *per se*.

The placental glycogen concentration also fell between 16 and 21 d.g. in N dams (Fig. 2). This decline was more pronounced between 16 and 19 d.g. in the maternal side than the fetal side, though glycogen concentration was higher in the former side at all stages, as expected (Ne'eman *et al.* 1987). Relative to N dams, glycogen concentration in total and maternal placenta from TX_s dams was elevated to a similar extent at 19 d.g. and at 21 d.g., whereas it was elevated in fetal placenta from TX_s dams at 16 and 19 d.g. (Fig. 2). Glycogen content showed a similar ontogeny to glycogen concentration in normal total and maternal placenta, and was similarly influenced

by maternal thyroidectomy (Fig. 2). In contrast, glycogen content in the fetal side increased ($P < 0.05$) slightly between 16 and 19 d.g. in N dams, as the mass of this tissue doubled (Table 2); treatment-associated changes were limited to an elevation in TX_s dams at 19 d.g. (Fig. 2).

Regression of the maternal placental glycogen concentration at 19 or 21 d.g. on the reciprocal of the pre-mating maternal serum TT₄ level revealed significant relationships (Fig. 3) of comparable slope but different ($P < 0.001$) elevation. The fetal placental glycogen concentration at 19 d.g. only was related to the reciprocal of the pre-mating maternal serum TT₄ level, albeit the data at 16 d.g. were highly scattered (Fig. 3). Since fetal size was reduced in TX dams, fetal demands on placental metabolic stores may be reduced, and this may in part explain the elevated maternal and fetal placental glycogen concentrations. However only the maternal placental glycogen concentration at 21 d.g. was related to fetal body weight ($r = -0.518$; $P = 0.006$; $n = 27$). Furthermore, multiple regression analysis of these data against the reciprocal of pre-mating maternal serum TT₄ and fetal body weight yielded a significant relationship ($R = 0.598$; $P = 0.005$; $n = 27$), but only the relationship between the maternal placental glycogen concentration and the reciprocal of pre-mating maternal serum TT₄ tended towards statistical significance ($P = 0.080$). Reduced fetal size is therefore unlikely to account for elevated placental glycogen stores in TX_s dams.

Serum and amniotic fluid glucose concentrations

The maternal serum glucose concentration was normal in TX dams at all stages (Table 4). Fetal serum glucose at 16 and 21 d.g. displayed hyperbolic relationships with pre-mating maternal serum TT₄ (Fig. 4), but levels were only reduced in TX_s dams at 21 d.g. (Table 4). The amniotic fluid glucose concentration at 16 d.g. also displayed a hyperbolic relationship with pre-mating maternal serum TT₄ (Fig. 4) and was reduced in TX_m and TX_s dams at this stage only (Table 4).

At 16 d.g., amniotic fluid glucose was related to fetal body weight ($r = 0.518$; $P = 0.007$; $n = 26$), as was the case at 21 d.g. for fetal serum glucose ($r = 0.599$; $P < 0.001$; $n = 37$). Upon multiple regression analysis, fetal serum glucose at 21 d.g. ($R = 0.599$; $P < 0.001$) was related to fetal body weight ($P = 0.001$) but no longer to the reciprocal of pre-mating maternal serum TT₄, whereas amniotic fluid glucose at 16 d.g. ($R = 0.606$; $P = 0.005$) failed to show a significant relationship with either fetal body weight or the reciprocal of pre-mating maternal serum TT₄ (albeit $P = 0.077$ and 0.071 respectively). Thus, in TX_s dams near term at least, the apparent fetal hypoglycemia is likely to arise secondary to fetal growth retardation, since the fetal serum glucose concentration appears normal for fetal body

Figure 2 Placental glycogen levels in N, TX_m and TX_s dams. Glycogen concentration (A–C) and content (D–F) are shown for total placenta (A and D), the maternal side (B and E) and the fetal side (C and F) from N (○), TX_m (△) and TX_s (□) dams. Results are expressed as means ± S.E.M. ^a $P < 0.05$, ^b $P < 0.02$, ^c $P < 0.01$, ^d $P < 0.005$, ^e $P < 0.002$ and ^f $P < 0.001$ TX_s vs N dam; [#] $P < 0.05$, ^{##} $P < 0.02$ and ^{###} $P < 0.01$ TX_s vs TX_m dam; $n \geq 4$ pregnancies for total placenta and $\geq$ a further six pregnancies for maternal/fetal placenta.

weight, albeit both parameters are depressed in TX_s dams relative to N dams.

Fetal growth retardation near term is unrelated to maternal and placental glucose metabolic compromise

The onset of fetal thyroid activity rapidly normalizes fetal tissue thyroid hormone pools in overtly hypothyroid rat dams (Ruiz de Ona *et al.* 1991), so that persisting fetal hypothyroidism cannot explain the pronounced fetal body weight deficits in TX_s dams near term. In this study, the observation in TX dams that fetal serum glucose levels appear normal for fetal body weight, and that maternal

serum glucose concentrations were normal, further suggests that neither placental nor maternal glucose metabolic compromise contribute to fetal growth retardation in hypothyroid pregnancies near term. To test this proposal, a subset ($n = 17$) of N and TX dam pregnancies at 21 d.g. was selected for which fetal body weight, litter size, pre-mating maternal serum TT₄, maternal serum glucose, maternal hepatic glycogen and maternal placental glycogen concentrations were all characterized. Multiple regression analysis was performed using a stepwise procedure with fetal body weight as the dependent variable and all other parameters (the reciprocal of pre-mating maternal serum TT₄ was used for this analysis) as predictor variables.

Figure 3 Relationship between placental glycogen concentration and pre-mating maternal serum TT₄ levels. Data are derived from N and TX dams at 16 d.g. (□), 19 d.g. (●) and 21 d.g. (△). (A) Regression of the glycogen concentration in the maternal side of the placenta on the reciprocal of TT₄ yields significant relationships at 19 d.g. ($r=0.411$; $P=0.030$; $n=28$) and at 21 d.g. ($r=0.543$; $P=0.004$; $n=27$) of similar slope (pooled slope=113.4) but different ($P=0.003$) elevation. (B) Regression of the glycogen concentration in the fetal side of the placenta on the reciprocal of TT₄ yields a significant relationship at 19 d.g. only (slope=49.7 ± 21.4; $r=0.415$; $P=0.028$; $n=28$).

This resulted in the selection of a one-factor multiple regression model ($R=0.671$; $P=0.003$) in which fetal body weight was related only to the reciprocal of pre-mating maternal serum TT₄. Consequently, maternal and/or placental glucose metabolic compromise are unlikely to contribute to fetal growth retardation in hypothyroid pregnancies near term.

Discussion

We have studied rat dams thyroidectomized several weeks before pregnancy and shown that the impact on fetal growth varies according to the severity of maternal hypothyroidism, the stage of pregnancy and the fetal tissue under study. Moderate hypothyroidism (pre-mating maternal serum TT₄ > 16.19 nM) was associated with transient reductions in fetal body and brain weights before the onset of fetal thyroid hormone secretion, and a

Table 4 Serum and amniotic fluid glucose levels in N, TX_m and TX_s dam pregnancies

		Maternal serum (mM)	Fetal serum (mM)	Amniotic fluid (mM)
16 d.g.	N	7.02 ± 0.28	2.23 ± 0.11	4.10 ± 0.16
	TX _m	7.04 ± 0.28	1.89 ± 0.18	3.67 ± 0.18 ^a
	TX _s	6.91 ± 0.19	1.83 ± 0.13	3.46 ± 0.05 ^b
19 d.g.	N	6.29 ± 0.20	1.40 ± 0.13	2.04 ± 0.18
	TX _m	6.04 ± 0.23	1.37 ± 0.18	1.89 ± 0.10
	TX _s	6.32 ± 0.21	1.28 ± 0.09	1.90 ± 0.13
21 d.g.	N	5.75 ± 0.23	5.06 ± 0.26	
	TX _m	5.78 ± 0.48	4.30 ± 0.49	
	TX _s	5.56 ± 0.33	3.56 ± 0.25 ^b	

^a $P<0.05$ and ^b $P<0.001$ TX_m or TX_s vs N dam; $n \geq 12$ different pregnancies for maternal serum, $n \geq 6$ different pregnancies for fetal serum and $n \geq 4$ different pregnancies for amniotic fluid. Rat amniotic fluid at 21 d.g. is very viscous and was not collected.

reduction in fetal liver weight near term. In contrast, more severe hypothyroidism was associated with deficits in fetal body, brain and liver weights which were more pronounced and asymmetrical near term; fetal liver weight was more markedly reduced than body weight, whereas some brain growth-sparing occurred. These findings are in agreement with earlier studies of dams made hypothyroid before pregnancy (Morreale de Escobar *et al.* 1985, Pickard *et al.* 1999). We hypothesized that maternal and placental glycogen storage and glucose supply to the fetus are disturbed in TX dams, and that such glucose metabolic compromise contributes to fetal growth retardation, especially near term, when the fetal demand for glucose is high.

Thyroidectomy of rat dams on the day of conception impairs the build-up of the maternal metabolic stores, including liver glycogen, required to support late fetal growth. Maternal hypoglycemia may (Bonet & Herrera 1991) or may not (Porterfield *et al.* 1975) ensue, however fetal and placental growth is severely retarded in late gestation (Bonet & Herrera 1988, Hendrich & Porterfield 1992). In contrast, in the present study, maternal liver glycogen concentration was elevated in TX_s dams at 16 and 19 d.g. This increase was partly related to pre-mating maternal thyroid status, in keeping with studies in thyroidectomized non-pregnant adult animals (Tata *et al.* 1963, Castro & Herrera 1973) and was also related to litter size, which was depressed in TX dams. Importantly, maternal euglycemia was maintained in TX_s dams, perhaps due to the establishment of compensatory metabolic mechanisms before exposure to the stress of pregnancy, and to the depression in litter size and thus feto-placental metabolic demands.

Rat placental glycogen stores are depleted by chronic T₃ administration in late gestation (Shafir *et al.* 1994). In agreement with this finding, placental glycogen

Figure 4 Relationship between fetal glucose levels and pre-mating maternal serum TT_4 . Data are derived from N and TX dams at 16 d.g. ($\square$), 19 d.g. ($\bullet$) and 21 d.g. ($\triangle$). (A) Regression of fetal serum glucose levels on the reciprocal of TT_4 yields significant relationships at 16 d.g. ($r=-0.551$; $P=0.010$; $n=21$) and 21 d.g. ($r=-0.402$; $P=0.017$; $n=35$). (B) Regression of amniotic fluid glucose levels on the reciprocal of TT_4 yields a significant relationship at 16 d.g. ($r=-0.522$; $P=0.006$; $n=26$).

concentration was elevated in TX_s dams: at 19 and 21 d.g. in the maternal side, but at 16 and 19 d.g. in the fetal side, independent of reduced fetal size. These differences may be cell specific, since glycogen is predominantly found in glycogen cells and syncytiotrophoblasts on the maternal and fetal sides respectively (Davies & Glasser 1968, Ne'eman *et al.* 1987). Placental thyroid hormone homeostasis may also be influential since, in hypothyroid dams, the $T_3:T_4$ ratio is increased on the maternal side, whereas fetal thyroid function compensates thyroid hormone deficits on the fetal side (Ruiz de Ona *et al.* 1991, Calvo *et al.* 1992). Thyroidectomy of pregnant dams, in contrast, causes no elevation in term placental glycogen

levels (Porterfield *et al.* 1975), perhaps due to the attendant severe maternal metabolic and placental growth compromise.

At 16 d.g., fetal serum and amniotic fluid glucose levels exhibited similar dependencies on pre-mating maternal serum TT_4 , but only the amniotic fluid glucose level was reduced in TX_m and TX_s dams. Previously, fetal serum glucose was found to be depressed in moderately hypothyroid dams at 16 d.g. (Pickard *et al.* 1999). The reason for this discrepancy is unclear, but current findings indicate that fetuses of TX dams exhibit at worst only mild hypoglycemia prior to the onset of fetal thyroid function. At 19 d.g., fetal serum and amniotic fluid glucose concentrations had normalized in TX_m and TX_s dams, but fetal liver glycogen stores were deficient. At 21 d.g., these normalized in TX_m dams, whereas they remained deficient in TX_s dams, and this was accompanied by severe fetal hypoglycemia. Deficient liver glycogen storage coupled with hypoglycemia is also characteristic of term fetuses from dams thyroidectomized soon after conception (Porterfield *et al.* 1975). In our model at least, such effects were strongly related to fetal body weight, pre-mating maternal serum TT_4 having no residual influence.

The relationship between fetal body weight and serum glucose may indicate that fetal body weight is strongly constrained by fetal serum glucose or, alternatively, that the ontogenic increase in fetal serum glucose concentration is dependent on some uncharacterized factor related to fetal growth. The data analysis presented here cannot discriminate unambiguously between these possibilities, since the statistical approach used does not allow mechanistic conclusions to be made definitively. The second possibility is perhaps more likely to occur in this model however, since the maternal serum glucose concentration and placental expression of GLUT protein isoforms (Pickard *et al.* 1999) are normal in TX dams near term, albeit materno-fetal glucose transport remains to be determined. Thus fetal growth retardation in TX_s dams at this stage is unlikely to be due to impaired fetal glucose availability or maternal/placental glucose metabolic compromise. Indeed, maternal liver and placental glycogen concentrations and maternal serum glucose were all rejected as predictor variables for fetal body weight at 21 d.g. in the multiple regression analysis. This was not unexpected for maternal liver glycogen and serum glucose levels, since both were normal in TX_s dams at 21 d.g. A role for maternal glucose metabolic compromise in fetal growth retardation in TX_s dams at earlier stages of pregnancy is also unlikely, since fetal body weight at 16 and 19 d.g. was unrelated to maternal serum glucose (data not shown) and hepatic glycogen concentrations.

More general disturbances in maternal metabolism may contribute to fetal growth retardation in TX dams, since hypothyroidism during pregnancy impairs maternal body weight gain (Bonet & Herrera 1988, Versloot *et al.* 1998). This did not appear to occur in the present study, since

although maternal carcass weight (after removal of uteri) tended to be slightly lower (by 10%) for TX_m and TX_s dams compared with N dams at 16 and 21 d.g. (data not shown), it had no influence on fetal growth in single or multiple regression analyses with other relevant factors (data not shown). Furthermore, maternal nutritional restriction depresses placental weight and glycogen stores (Woodall *et al.* 1996, Rudge *et al.* 1999), whereas the TX_s dams in the present study exhibited normal placental weight and elevated placental glycogen stores.

In conclusion, fetal body weight during late gestation exhibits a hyperbolic relationship with pre-mating maternal serum TT₄ levels in rat dams made hypothyroid before conception; permanent fetal growth retardation occurring only in severely hypothyroid dams. In such pregnancies, excessive glycogen storage occurs in the maternal liver and the placenta from before the onset of fetal thyroid function, whereas fetal liver glycogen stores and serum glucose levels are depressed near term. These fetal deficits appear due to the attendant fetal growth retardation, and neither maternal nor placental glucose metabolic compromise are likely to contribute to this process near term. The mechanisms by which severe maternal hypothyroidism retard fetal growth near term remain to be determined.

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