Effects of pregnancy and lactation on plasma lipid and lipoprotein concentrations, lipoprotein composition and post-heparin lipase activities in Shetland pony mares

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The incidence of hyperlipaemia in ponies is highest in mares in late gestation and then early in lactation. Plasma lipid and lipoprotein concentrations were measured to establish the metabolic basis for this and the lipoprotein composition of six healthy Shetland ponies was analysed before pregnancy, in the last six weeks of gestation and one month after foaling. In the pregnant ponies, the concentrations of cholesterol and triglyceride were significantly increased (both $P < 0.05$) because of increased concentrations of high density lipoproteins (HDL) and very low density lipoproteins (VLDL), respectively (both $P < 0.05$). The VLDL were significantly enriched in triglyceride and depleted of protein ($P < 0.05$ in each case), with the majority of cholesterol in the free rather than esterified form. These changes appeared to reflect increased hepatic triglyceride synthesis and VLDL secretion because the activities of lipoprotein lipase and hepatic lipase, the enzymes responsible for the catabolism of VLDL and their remnants, were unaltered. After foaling, the concentrations of triglyceride and VLDL decreased significantly (both $P < 0.05$) because of increased lipoprotein lipase activity consistent with the induction of the enzyme in mammary tissue to provide for milk fat synthesis. Plasma cholesterol and HDL concentrations remained high and non-esterified fatty acid concentrations were significantly increased ($P < 0.05$). The VLDL remained enriched in triglyceride but had normal cholesterol and protein compositions, although the mass of phospholipids was reduced. The changes in plasma VLDL concentration and composition found in the pregnant ponies mimicked those previously reported in ponies with hyperlipaemia and suggested a metabolic basis for the incidence of the disease in late gestation.

Introduction

Hyperlipaemia is a disorder of lipid metabolism peculiar to the pony breeds of horse (Schotman and Wagenaar, 1969) and donkeys (Fowler, 1989). Blood from affected animals is grossly lipaemic and there is fatty infiltration of body tissues leading to organ failure and death (Jeffcott and Field, 1985a). The lipaemia is accompanied by high plasma concentrations of triglyceride and cholesterol due to increased concentrations of very low density lipoproteins (VLDL) (Morris *et al.*, 1972; Bauer, 1983). Although the metabolic origins of this hyperlipidaemia are unclear, defective clearance of VLDL from the circulation has been suggested to be the cause (Morris *et al.*, 1972; Bauer, 1983; Freestone *et al.*, 1991). However, this might not be the case, as the activity of lipoprotein lipase, the rate-limiting enzyme in the catabolism of VLDL, is not reduced in post-heparin plasma from ponies with hyperlipaemia (Bauer *et al.*, 1987; Watson *et al.*, 1990a). Indeed, the chemical composition of VLDL from affected animals suggests that the hyperlipidaemia arises from overproduction of VLDL by the liver (Watson *et al.*, 1992a).

In their initial description of the disease, Schotman and Wagenaar (1969) reported that more than 90% of the 138 cases of hyperlipaemia that they saw over seven years were mares that were in foal, or had recently foaled or aborted. This association with reproductive events has since been confirmed in Australia where, in a series of 30 cases that included 27 mares (90%), 15% of the mares were pregnant and 80% lactating (Jeffcott and Field, 1985b). In a series of 18 cases in the United Kingdom that included 14 mares (78%), 64% of the mares were in foal (range 5–10 months gestation; mean $\pm$ SD, 8.4 ± 1.6 months) and 14% were lactating, having foaled between one and six weeks before the onset of disease (Watson *et al.*, 1992b).

A plausible explanation for the association of this disease with pregnancy and lactation comes from the hypothesis that hyperlipaemia arises from the mobilization of fatty acids from adipose tissue in response to a negative energy balance (Naylor *et al.*, 1980). Thus the increased demands placed on maternal energy supplies by fetal growth and milk production could induce adipose lipolysis leading to a greater flux of non-esterified fatty acids (NEFA) to the liver, increased hepatic tri-

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glyceride synthesis and overproduction of VLDL (Jeffcott and Field, 1985a). In addition, this response may be exacerbated in late gestation by the development of peripheral insensitivity to the action of insulin (Fowden *et al.*, 1984).

Despite this evidence, the impact of pregnancy and lactation on maternal plasma lipid and lipoprotein metabolism has received little attention. The association of hyperlipaemia with these states was examined by measuring plasma lipid and lipoprotein concentrations in six Shetland ponies before they were put in foal, in the last trimester of pregnancy and again one month after foaling. The chemical composition of the major lipoprotein classes was determined and the activities of lipoprotein lipase and hepatic lipase, the enzymes responsible for the catabolism of VLDL and their remnants, were measured.

Materials and Methods

Animals and treatments

Blood samples were collected from six healthy adult Shetland pony mares one week after the ponies were housed in pairs in loose boxes before running at grass with a Shetland stallion. All had previously had foals and their body weights ranged from 182–249 kg (mean $\pm$ SD; 220 ± 27 kg). They were housed again in pairs approximately one month before their estimated foaling dates and blood samples taken one week later, which was between one and five weeks before they actually foaled. All mares gave birth to mature, full-term foals; they were then kept in individual loose boxes and blood samples collected one month after foaling. Throughout all of the housed periods, the mares were fed hay *ad libitum*.

All blood samples were collected by jugular venepuncture after an overnight fast. Blood for lipid and lipoprotein analysis was collected into potassium EDTA (final concentration 1 mg ml^{-1}); plasma was separated by centrifugation at 1550 g for 15 min and stored at 4°C before analysis. Blood for lipoprotein lipase and hepatic lipase measurement was collected 10 min after i.v. injection of heparin at a dose of 70 iu kg^{-1} body weight; the samples were placed in ice and the plasma separated at 4°C and stored at -20°C before analysis.

Plasma lipid and lipoprotein concentrations

Triglyceride and total cholesterol were measured using reagent kits (704161, 237574; Boehringer Mannheim UK Ltd, Lewes) on an automated discrete analyser (Boehringer Mannheim/Hitachi 717; Boehringer Mannheim UK Ltd, Lewes). Plasma lipoprotein cholesterol concentrations were determined by a combined ultracentrifugation/precipitation technique (Watson *et al.*, 1991). Plasma NEFA were measured using an enzymatic colorimetric kit (NEFA C, Wako Chemicals GmbH; Alpha Laboratories Ltd, Maidstone).

Lipoprotein composition

Very low density lipoproteins, low density lipoproteins (LDL) and high density lipoproteins (HDL) were isolated from 4 ml plasma by sequential flotation ultracentrifugation at densities of

$<1.006 \text{ g ml}^{-1}$, $1.019\text{--}1.063 \text{ g ml}^{-1}$ and $1.063\text{--}1.225 \text{ g ml}^{-1}$, respectively (Havel *et al.*, 1955). The triglyceride and total cholesterol contents of each lipoprotein fraction were measured as for plasma triglyceride and cholesterol. Free cholesterol and phospholipid were determined using enzymatic colorimetric kits (310328, 691844; Boehringer Mannheim UK Ltd, Lewes) and total protein was measured by the modified method of Lowry *et al.* (Peterson, 1977).

Lipoprotein lipase and hepatic lipase activity

The activities of lipoprotein lipase and hepatic lipase were measured in plasma samples taken 10 min after heparin administration using a selective assay system (Watson *et al.*, 1992c). In brief, aliquots of plasma were incubated in duplicate at 28°C for 60 min with a gum arabic stabilized triglyceride emulsion containing glycerol tri[$1\text{-}^{14}\text{C}$]oleate (CFA 258; Amersham International plc, Amersham). The fatty acid products of lipolysis were captured by BSA, extracted from the glycerides and radioactivity was measured by liquid scintillation counting. The activity of hepatic lipase was measured in the presence of $1.0 \text{ mol NaCl l}^{-1}$, under which conditions the activity of lipoprotein lipase was negligible ($<8\%$). The activity of lipoprotein lipase was measured at $0.1 \text{ mol NaCl l}^{-1}$ after inhibition of hepatic lipase ($>93\%$) by prior incubation with $50 \text{ mmol SDS l}^{-1}$. The results were expressed in $\mu\text{mol fatty acids released per ml of plasma per hour of incubation}$ ($\mu\text{mol FA ml}^{-1} \text{ h}^{-1}$).

Statistical analysis

Two group comparisons i.e. pregnancy–pre-pregnancy, lactation–pre-pregnancy, lactation–pregnancy were made by paired analysis using the Wilcoxon signed-rank test.

Results

Plasma lipid and lipoprotein concentrations

The plasma concentrations of cholesterol and triglyceride were significantly higher in the ponies in late gestation than before pregnancy, with the mean values increased over those before pregnancy by 47 and 162%, respectively (both $P < 0.05$; Table 1). This was due to significant increases in the concentrations of VLDL- and HDL-cholesterol, the mean values of which were increased by 28 and 53%, respectively (both $P < 0.05$). There was no significant change in the concentrations of LDL-cholesterol or NEFA.

After foaling, the cholesterol concentrations remained high but were not statistically different from the pre-pregnancy values. The concentrations of triglyceride and VLDL-cholesterol decreased significantly (both $P < 0.05$); the triglyceride concentrations returned to basal values, but those of VLDL became significantly lower than before pregnancy ($P < 0.05$). There was no change in LDL- or HDL-cholesterol concentrations, and the latter remained significantly higher than the concentration before pregnancy ($P < 0.05$). Plasma NEFA concentrations increased significantly after foaling ($P < 0.05$).

Table 1. Plasma cholesterol, triglyceride, lipoprotein-cholesterol and non-esterified fatty acid concentrations in six Shetland pony mares before pregnancy, in late gestation and in early lactation

Animal	Plasma concentration (mmol l ⁻¹)					
	Cholesterol	Triglyceride	VLDL	LDL	HDL	NEFA
Pre-pregnancy	1.98 ± 0.29	0.52 ± 0.10	0.43 ± 0.08	0.48 ± 0.23	1.13 ± 0.20	0.49 ± 0.19
Pregnant	2.91 ± 0.25 ^a	1.36 ± 0.49 ^a	0.55 ± 0.11 ^a	0.63 ± 0.12	1.73 ± 0.22 ^a	0.43 ± 0.20
Lactating	2.78 ± 0.76	0.41 ± 0.27 ^b	0.23 ± 0.09 ^{ab}	0.77 ± 0.70	1.75 ± 0.27 ^a	0.98 ± 0.48 ^b

Values are means ± SD.

^aSignificantly different ($P < 0.05$) from pre-pregnancy values.

^bSignificantly different ($P < 0.05$) from pregnant values.

VLDL: very low density lipoproteins; LDL: low density lipoproteins; HDL: high density lipoproteins; NEFA: non-esterified fatty acids.

Table 2. Chemical composition of very low density lipoproteins from Shetland pony mares before pregnancy, in late gestation and in early lactation

Animal	Lipoprotein (percentage particle mass)				
	Cholesteryl esters	Triglyceride	Free cholesterol	Phospholipid	Protein
Pre-pregnancy	8.8 ± 0.9	60.4 ± 1.7	ND	16.6 ± 2.3	16.4 ± 2.0
Pregnant	0.7 ± 0.6 ^a	68.5 ± 2.1 ^a	7.8 ± 1.8	15.0 ± 0.9	8.0 ± 1.5 ^a
Lactating	6.6 ± 1.6 ^b	67.8 ± 4.7 ^a	ND	9.5 ± 1.4 ^{ab}	16.2 ± 3.6 ^b

Values are means ± SD.

ND: not detected.

^aSignificantly different ($P < 0.05$) from pre-pregnancy values.

^bSignificantly different ($P < 0.05$) from pregnant values.

Table 3. Chemical composition of low density lipoproteins from Shetland pony mares before pregnancy, in late gestation and in early lactation

Animal	Lipoprotein (percentage particle mass)				
	Cholesteryl esters	Triglyceride	Free cholesterol	Phospholipid	Protein
Pre-pregnancy	41.2 ± 2.6	5.9 ± 2.5	1.2 ± 1.9	21.4 ± 1.6	29.1 ± 3.8
Pregnant	37.8 ± 3.5 ^a	5.9 ± 1.2	8.0 ± 4.3 ^a	24.6 ± 1.9	23.1 ± 2.8 ^a
Lactating	34.5 ± 9.1	4.5 ± 2.0	9.8 ± 5.6 ^a	27.3 ± 8.1	25.5 ± 6.3 ^a

Values are means ± SD.

^aSignificantly different ($P < 0.05$) from pre-pregnancy values.

Lipoprotein composition

The VLDL from the pregnant mares contained significantly more triglyceride, and less protein, than the particles isolated before pregnancy ($P < 0.05$ in each case; Table 2). Although the total cholesterol content of VLDL was unchanged, the mass of cholesteryl esters was significantly reduced ($P < 0.05$) and free cholesterol, which was not detected in VLDL from the ponies before pregnancy, accounted for approximately 8% of

particle mass and represented 92% of the total cholesterol mass in VLDL. After foaling, the VLDL remained enriched in triglyceride, but the mass of protein was significantly reduced ($P < 0.05$) returning to basal values. The mass of cholesteryl esters increased significantly ($P < 0.05$), returning to the amounts found before pregnancy, and no free cholesterol was detected. The mass of phospholipid in VLDL from the lactating mares was significantly lower than before and during pregnancy ($P < 0.05$ in each case).

Table 4. Chemical composition of high density lipoproteins from Shetland pony mares before pregnancy, in late gestation and in early lactation

Animal	Lipoprotein (percentage particle mass)				
	Cholesteryl esters	Triglyceride	Free cholesterol	Phospholipid	Protein
Pre-pregnancy	19.6 ± 3.4	1.0 ± 0.4	3.3 ± 4.1	31.0 ± 4.1	45.3 ± 6.8
Pregnant	20.9 ± 3.4	0.4 ± 0.1 ^a	4.1 ± 0.8	30.4 ± 1.2	44.2 ± 3.2
Lactating	20.7 ± 2.2	0.6 ± 0.2	4.0 ± 0.4	28.1 ± 3.4	49.9 ± 4.8

Values are means ± SD.

^aSignificantly different ($P < 0.05$) from pre-pregnancy values.

Table 5. Lipoprotein lipase and hepatic lipase activities in six Shetland pony mares before pregnancy, in late gestation and in early lactation

Animal	Lipoprotein lipase ($\mu\text{mol FA ml}^{-1} \text{h}^{-1}$)	Hepatic lipase ($\mu\text{mol FA ml}^{-1} \text{h}^{-1}$)
Pre-pregnancy	3.44 ± 1.10	5.05 ± 1.88
Pregnant	4.12 ± 0.68	3.93 ± 0.83
Lactating	14.76 ± 4.44 ^{ab}	9.78 ± 3.83 ^{ab}

Values are means ± SD.

^aSignificantly different ($P < 0.05$) from pre-pregnancy values.

^bSignificantly different ($P < 0.05$) from pregnant values.

FA: fatty acids released

Changes in LDL composition in late pregnancy included a significant reduction in the masses of cholesteryl esters and protein, and a significantly increased mass of free cholesterol (all $P < 0.05$; Table 3). There was no change in LDL composition after foaling and the masses of triglyceride and phospholipid were unaffected by pregnancy or lactation.

The mass of triglyceride in HDL was significantly reduced in the pregnant ponies ($P < 0.05$), but increased after foaling so that it was not significantly different from the pre-pregnancy values (Table 4). The masses of cholesteryl esters, free cholesterol, phospholipids and protein in HDL were unaltered during pregnancy and lactation.

Lipoprotein lipase and hepatic lipase

The activities of lipoprotein lipase and hepatic lipase in the pregnant ponies were similar to those found before pregnancy (Table 5). After foaling, the activities of both enzymes increased significantly, by on average 329% (lipoprotein lipase) and 94% (hepatic lipase) over basal values ($P < 0.05$ in each case).

Discussion

This study, although using only a small number of animals, showed that plasma lipid metabolism was altered in pony mares

in late gestation. Most significantly, the concentrations of triglyceride were increased, because of high VLDL concentrations, so that in four of the six ponies, plasma triglyceride concentrations (1.20, 1.20, 1.75, 2.15 mmol l^{-1}) were greater than the upper limit of the reference range (0.99 mmol l^{-1}). This result is consistent with the findings of Stammers *et al.* (1989) who reported triglyceride concentrations in the uterine vein in excess of 1.0 mmol l^{-1} in two (1.33 and 5.53 mmol l^{-1}) of three healthy pony mares sampled between 250 and 300 days of gestation. These two sets of data indicate that moderate hypertriglyceridaemia is prevalent in late gestation in pony mares. Although mean plasma cholesterol concentrations were increased in the pregnant ponies, none of the individual values was greater than the upper limit of our reference range. The increased cholesterol concentrations were largely accounted for by the rise in HDL-cholesterol. This is in contrast to the moderate hypercholesterolaemia found in ponies with hyperlipaemia which is due to high VLDL-cholesterol concentrations, as the concentrations of HDL are normal (Watson *et al.*, 1992a).

The hypertriglyceridaemia found in the pregnant ponies was less severe than that seen in ponies with hyperlipaemia where triglyceride concentrations are in the range 4.7–78.8 mmol l^{-1} (Watson *et al.*, 1992b) with reported mean values of 16.5 mmol l^{-1} (Naylor *et al.*, 1980), 25.4 mmol l^{-1} (Watson *et al.*, 1992b) and 39.2 mmol l^{-1} (Eriksen and Simesen, 1970). Despite this difference, the chemical composition of VLDL in the pregnant ponies was almost identical to that of VLDL found in ponies with hyperlipaemia (Watson *et al.*, 1992a). The particles were enriched in triglyceride and depleted of protein, and although the total mass of cholesterol was unchanged, the mass of free cholesterol was increased whereas that of cholesteryl esters was reduced. These alterations are important because they reflect the metabolic origins of hyperlipaemia and indicate that similar events may be occurring in late gestation. With the absence of cholesteryl ester transfer protein activity in the horse (Watson *et al.*, 1993), these changes are consistent with the production by the liver of large VLDL particles that accommodate a greater mass of triglyceride by having apoB-48 rather than apoB-100 as their structural protein (Watson *et al.*, 1992a). In this way, the liver appears to maximize the secretion of triglyceride in response to increased hepatic triglyceride synthesis.

The appearance in VLDL of free cholesterol at the expense of cholesteryl esters reflects a reduction in the esterification of cholesterol. This could be related to a reduction in the activity

of the plasma cholesterol esterifying enzyme lecithin:cholesterol acyl transferase (LCAT). However, this might not be the case as the masses of esterified and free cholesterol were unchanged in HDL, which is the prime focus of LCAT activity in horses (Yamamoto *et al.*, 1975), in the ponies in the study reported here and in ponies with hyperlipaemia (Watson *et al.*, 1992a) in which the activity of LCAT has been shown to be normal (Watson, 1991). It is therefore more likely that the reduction in VLDL cholesteryl esters results from a decrease in the activity of the hepatic esterifying enzyme acyl CoA cholesterol acyl transferase relative to an increased rate of VLDL production.

We (Watson *et al.*, 1992a) and others (Naylor *et al.*, 1980; Bauer and Ransone, 1983; Jeffcott and Field, 1985a) have suggested that the stimulus for hepatic triglyceride synthesis, and hence the overproduction of VLDL in ponies with hyperlipaemia, is an increased flux of NEFA to the liver from peripheral adipose stores. This hypothesis is supported by the observation that plasma NEFA concentrations have been found to be high in hyperlipaemic ponies (Naylor *et al.*, 1980; Watson *et al.*, 1992a). Although NEFA concentrations were not high in the pregnant ponies here or in the study reported by Stammers *et al.* (1989), it is possible that the flux of NEFA to the liver was increased. The discrepancy between plasma NEFA concentration and flux is illustrated by the fact that the flux of NEFA to the liver is increased 32-fold in ponies with hyperlipaemia, whereas plasma NEFA concentrations are increased only five fold (Watson, 1991).

After foaling, the plasma cholesterol and triglyceride concentrations returned to the values seen before pregnancy. The VLDL concentrations, however, became significantly lower than those in the ponies before pregnancy; this can be explained by the increased activity of lipoprotein lipase. In other species this is due to the induction of the enzyme in mammary tissue by prolactin and provides the mechanism by which NEFA are channelled, via VLDL triglyceride, to the mammary gland for milk fat synthesis (Scow and Chernick, 1987). The rise in plasma NEFA concentrations that was seen after foaling might therefore have been due to increased lipolysis of VLDL triglyceride. Alternatively, it could have reflected mobilization of NEFA from adipose tissues to meet the increased demands placed by milk production on maternal energy metabolism. Although the VLDL from the lactating mares remained enriched in triglyceride, its cholesterol and protein composition was similar to that before pregnancy and was distinct from that found during pregnancy and in ponies with hyperlipaemia, suggesting that VLDL production was not stimulated to the same extent.

Apart from the changes in the concentration and composition of VLDL, there were changes in LDL and HDL that require explanation. The increased free cholesterol content of LDL in the pregnant ponies may be related to diminished LCAT activity or have reflected the fact that these particles are formed from VLDL. However, after foaling, the LDL remained depleted of cholesteryl esters and enriched in free cholesterol even though the cholesterol composition of VLDL had returned to normal. The reduced mass of phospholipid in VLDL in the lactating ponies could be explained by the increased activity of hepatic lipase, which has significant phospholipase activity (Ehnholm *et al.*, 1975). The reason for the increase in HDL concentrations during pregnancy and lactation is less obvious. In healthy, non-pregnant ponies the plasma concentrations of

HDL are positively correlated with those of triglyceride (Watson *et al.*, 1993) and it is possible that the high HDL concentrations were related to coordinate upregulation of hepatic HDL and triglyceride synthesis during pregnancy and lactation. The increased HDL concentrations may also reflect increased transport of cholesterol to steroidogenic tissues during these periods, for example to provide the rapid increase in circulating progestagens that commences in the mare at about 300 days of gestation and persists until parturition (Arthur *et al.*, 1982).

In summary, we have found significant changes in plasma triglyceride and VLDL concentrations, and in the composition of VLDL, in pony mares during the last trimester of gestation that mimic those found in ponies with hyperlipaemia. These changes are consistent with the mobilization of NEFA from adipose tissue and increased hepatic triglyceride synthesis and VLDL secretion. This may explain why hyperlipaemia is most prevalent in ponies in late gestation and reflect the sensitivity of pregnant mares to disturbances in energy balance. This was evidenced by the greatly increased triglyceride concentration (12.7 mmol l^{-1}) that Stammers *et al.* (1989) found in one pony mare on a low plane of nutrition and the hypertriglyceridaemia (10.6 mmol l^{-1}) that they induced in one pregnant pony mare by withholding food for 30 h. With this in mind, attention should be focused on the nutrition of the pregnant pony and the presence of other factors, such as stress and obesity, that modify insulin (Jeffcott *et al.*, 1986) and lipid metabolism (Watson *et al.*, 1990b) and might precipitate hyperlipaemia. In contrast, the pattern of plasma lipids and lipoproteins after foaling could not readily explain the incidence of hyperlipaemia in lactating mares.

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