Association of Backfat Thickness with Postheparin Lipoprotein Lipase Activity and Very Low Density Lipoprotein-Subfractions in Growing Pigs

T. C. Loh*, I. J. Lean[1] and P. F. Dodds[1]

Department of Animal Science, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

ABSTRACT : Sixteen pigs from 2 distinct genetic lines (LGAH and VFIL) obtained after eight generations of divergent selection for high (H) and low (L) lean tissue growth rate with *ad-libitum* feeding (LGA) and voluntary feed intake (VFI), respectively, were used in this study. The objectives of this investigation were to establish appropriate working conditions for the postheparin plasma lipoprotein lipase (LPL) assay and to study relationships between fat deposition and plasma lipids, very low density lipoprotein (VLDL) lipids, VLDL-subfractions and postheparin plasma LPL activity in growing pigs. Four preliminary experiments were performed to determine the appropriate working conditions for the postheparin plasma LPL assays. Postheparin plasma preincubated with SDS (20-50 mM) at 26°C for 45 minutes inhibited hepatic lipase activity. A total of 2 µl VLDL/assay produced maximum stimulation of LPL activity. Postheparin plasma protein and increasing incubation time contributed an optimum response. LGAH pigs had a significantly higher proportion subfraction 2 than VFIL pigs. No differences were observed in postheparin plasma LPL activity and backfat thickness for two lines of pigs. There were positive correlations between backfat thickness and proportion of subfractions 2 and postheparin plasma LPL activity but the results were not statistically significant. Backfat thickness was not statistically correlated with proportion of subfraction 2 and postheparin plasma LPL activity in a multiple regression analysis. It is believed that the apolipoprotein E, which is present in higher quantities in VLDL-subfraction 2 plays an important role for clearing VLDL triacylglycerol into adipose tissue. LPL activity of pigs can be measured by using postheparin plasma technique. If the relationships of backfat thickness and VLDL-subfraction 2 and postheparin plasma LPL activity can be established, it suggests that these parameters could be used as indicators in selection programmes. Further experiments need to be conducted by using larger sample size and different breed of pigs with greater differences in backfat thicknesses to confirm these trends. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 11 : 1592-1597)*

Key Words : Backfat Thickness, Very Low Density Lipoprotein, VLDL-Subfractions, Lipoprotein Lipase, Postheparin Plasma

INTRODUCTION

Very low density lipoproteins (VLDL) are the major lipoprotein particles produced by the liver and most of them are of hepatic origin, although some VLDL are produced in the enterocytes. They are vehicles which transport triacylglycerol from liver to extrahepatic tissues, mainly in adipose tissue, cardiac muscle, lung and lactating mammary gland, which either store triacylglycerol or use it as a source of energy or of intermediates for further synthesis (Fielding and Fielding, 1991). An enzyme known as lipoprotein lipase (LPL) is responsible for hydrolysis of plasma lipoprotein triacylglycerols. The reaction is facilitated by apolipoprotein C-II which associates with triacylglycerol-rich lipoprotein after they are secreted into plasma (Vainio et al., 1983). The enzyme plays an important role not only in the oxidation of triacylglycerol and fatty acids (e.g. heart, lung, skeletal muscle) but also in the resynthesis of triacylglycerol for storage (e.g. adipose tissue).

Adipose triacylglycerol is mobilised during late pregnancy in humans and rats: the liberated fatty acids are incorporated into VLDL by the liver and secreted into the blood stream. Similar hypertriacylglycerolaemia was also found in late-pregnant sows (Wright et al., 1995). It has been shown that VLDL can be separated into two subfractions by heparin-agarose chromatography (Shelburne and Quarfordt, 1977; Trezzi et al., 1983; Huff and Telford, 1984; Herrera et al., 1988; Evans et al., 1989; Fielding et al., 1989; Wright et al., 1995). Subfraction 1 contains greater concentrations of apolipoprotein C-II and acts as activator of extrahepatic LPL, whereas subfraction 2 contains higher quantities of apolipoprotein E. It is believed that apolipoprotein E plays an important role in anchoring VLDL to the heparan sulphate proteoglycan-bound LPL. The proportion of VLDL-subfraction 2 (higher affinity for heparin) from pregnant sows was related to piglet mortality (Wright et al., 1995). The objectives of this investigation were to establish appropriate working conditions for the postheparin plasma LPL assay and to extend the study to seek relationships between fat deposition and VLDL lipids, VLDL-subfraction 2 and postheparin plasma LPL activity in growing pigs. As most of the fat deposited in the adipose tissue of growing pigs is derived from the plasma lipoprotein (Kris-Etherton and Etherton, 1982), it seemed likely that the rate of fat deposition would be influenced by the concentration and nature of available lipoprotein in the plasma.

* Corresponding Author: Loh Teck Chwen. Tel: +603-89486101 3630, Fax: +603-89488246, E-mail: tcloh@agri.upm.edu. my

[1] Imperial College at Wye, University of London, Wye, Ashford, Kent, TN25 5AH, UK.

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MATERIAL AND METHODS

Animals

Sixteen Landrace pigs from two different genetic lines (LGAH and VFIL) after eight generations of divergent selection for high (H) and low (L) lean tissue growth rate with *ad libitum* feeding (LGA) and voluntary feed intake (VFI), respectively, (Cameron and Curran, 1994) were used in this experiment. The piglets were weaned at 30-32 days of age and maintained for 7 weeks after weaning. Pigs were individually penned and fed *ad-libitum*. Same diet was provided to all the pigs (table 1). At the end of the experiment, backfat thickness was measured ultrasonically (MEDATA, U.K.) at a point 6.5 cm off the midline of the mid-back (analogous to P2 carcass measurement) and in the shoulder and the loin areas.

Blood samples

At the end of experiment, the animals were fasted overnight and bled prior to the morning feed. Blood samples were collected from the jugular vein as described by Muirhead (1981). Plasma was isolated and then VLDL was prepared from it by ultracentrifugal floatation (Wright et al., 1995). Protein (Sigma Chemical Company Ltd., Poole, Dorset; procedure no. P5656), triacylglycerol (Biomen Ltd., Croydon, Surrey), total cholesterol (Biomen Ltd., Croydon, Surrey), free cholesterol (Boehringer Corporation, East Sussex) and phospholipid (Boehringer Corporation, East Sussex) concentrations of VLDL and plasma were determined using the appropriate diagnostic kits. VLDLs were subfractionated according to their affinity for heparin on a column of heparin-agarose (heparin-Sepharose CL-6B, obtained from Pharmacia Biosystems Ltd., Milton Keynes, Buckinghamshire) according to the method of Wright et al. (1995).

Table 1. Compositions of diet for all the pigs

	Basal diet
Ingredients	
Corn	250
Soybean meal	130
Fish meal	60
Barley	250
Vegetable oil	10
Limestone	5
Salt	1
Dicalsium phosphate	4
Vitamin mixture[1]	11.6
L-lysine HCl	1.5
Choline	1
DL-methionine	0.7
Analysed chemical compositions	
Crude protein, %	22
Digestible energy (MJ/kg)	14.72

[1]Vitamin mixture supplied (per kg of diet): retinal, 6.0 mg; choleccalciferol, 75 μg; D-α-tocopherol, 40 mg; mendione, 5.0 mg; thiamine, 4.0 mg; riboflavin, 6.4 mg; pyridoxine, 4.0 mg; cyanocobalimin, 40 μg; ascorbic acid, 50 mg; D-pantothenic acid, 19.2 mg; niacin, 40.0 mg; biotin, 0.1 mg; folacin, 0.8 mg and olaquindox, 50 mg.

Preparation of postheparin plasma

The pigs were fasted overnight and heparin was injected intravenously through the jugular vein at a dose of 60 IU per kg body weight (Bengtsson-Olivecrona and Olivecrona, 1992). Blood samples were collected via jugular vein after 15-20 minutes and collected into 2 plain vacutainer tubes (without anticoagulant). The blood samples were transported in a polystyrene box, filled with ice, to the laboratory for further processing. The postheparin plasma was obtained by centrifugation of the blood for 30 minutes at 3,000 rpm on a bench centrifuge. The postheparin plasma was retained and transferred to another clean test tube. The protein concentrations of postheparin plasma were determined by Biuret method (Plummer, 1971). The protein concentration of postheparin plasma was adjusted to 0.30 mg per assay prior to the determination of LPL activity.

Postheparin plasma was pre-treated with sodium dodecyl sulphate (SDS) in order to eliminate hepatic lipase (Baginsky, 1981). For this purpose, 0.5 ml of the postheparin plasma samples were pipetted into small test tubes containing 0.5 ml of 0-50 mM SDS in 0.2 M tris-HCl buffer, pH 8.2. These mixtures were then incubated at 26°C for 45 minutes before assay.

Preparation of [^{3}H] trioleoylglycerol

[^{3}H] trioleoylglycerol emulsion was prepared using the method of Nilsson-Ehle and Schotz (1976). It was purified with amberlite resin IR-45 (OH) in a column to remove any free fatty acids (Baginsky, 1981) which otherwise cause high blank values and to remove partial glycerides. Three volumes of glass-distilled toluene were used to wash the column before 50 μl of the undiluted radioactive source was injected onto the top of the resin bed. The column was eluted with toluene and the eluate was collected in 5 ml fractions. 2 μl of fractions were mixed with 10 ml of 'Parkard Scintillator 299^{TM}' in a glass vial to measure the radioactive content. Only the rapidly eluting fractions containing significant radioactivity (≈32 μCi/ml) were pooled. The remaining procedures were followed in accordance with the methods of Dodds et al. (1987). A volumn equivalent to 135 μCi was dispensed into a clean vial using a glass pipette and dried under a gentle stream of nitrogen. A stock solution of non-radioactive trioleoylglycerol was prepared in choloroform and adjusted to 180 mg. The equivalent of 10.8 mg phosphatidyl choline dissolved in chloroform was also dispensed into the vial and

the solvents were dried down under nitrogen. 3 ml of glycerol was added to the contents by weight and the mixture was dispensed by using Soniprep 150 (MSE, Crawley, West Sussex, UK). Thin layer chromatography coated with silica gel was used to check the purity of the emulsion. 10 µl of the emulsion was pipetted into a conical tube, followed by 0.5 ml distilled water and 1.88 ml chloroform/methanol (1:2 v/v) solution with subsequent mixing in accordance with the method of extraction by Bligh and Dyer (1959). The purity of the sample was evaluated by calculating the proportion of the total number of disintegration per minute (dpm) present in the sections corresponding to the position of trioleoylglycerol. This value was always found to exceed 91% with no appreciable radioactivity detectable in sections relating to the position of free fatty acids.

The concentration and specific activity of trioleoylglycerol emulsion were 67.8 mM and 0.51 mCi per mmol respectively. The emulsion was stored in the dark at room temperature. Before each assay, a 'working solution' of [^{3}H]-trioleoylglycerol was prepared by mixing 100 µl of emulsion (dispensed by weight), 700 µl of 0.429 M tris pH8.00, 185 µl of BSA (200 mg per ml, fatty acid free) from a concentrated solution and the final volume was adjusted to 1 ml with distilled water (Dodds et al., 1987).

Postheparin plasma lipoprotein lipase activity assay

Preliminary experiments were carried out to establish appropriate working conditions for the assay. In each experiment, 'time-zero' controls were prepared by the addition of 3.25 ml of chloroform/methanol/heptane (125:141:100 v/v/v) to 'stop the reaction' first before adding the activator and the postheparin plasma.

Each assay mixture contained 50 µl of [^{3}H]-trioleoylglycerol (working solution), 20 µl of VLDL as activator, a specified volume of postheparin plasma (adjusted to 0.30 mg per assay) and the final volume was adjusted to 200 µl with distilled water. The final concentrations in the assay were 1.7 mM trioleoylglycerol (0.51 mCi per mmol) and 75.1 mM tris (Dodds et al., 1987). The reaction was started by the addition of VLDL, followed 10 seconds later by the postheparin plasma. The assay tube was gently shaken to mix the contents and then incubated in a shaking water bath at 37°C for 30 minutes. The reaction was stopped by the addition of 3.25 ml of chloroform/methanol/heptane (125:141:100, by volume), followed by the addition of 1.05 ml of 0.1 M K_2CO_3/borate buffer (pH10.5) (Belfrage and Vaughan, 1969). The mixture was agitated on a vortex mixer, the upper phases were separated in a bench centrifuge for 10 minutes at 3,000 rpm. 1 ml of the upper phase was carefully transferred to a scintillation vial, to which 100 µl of 2.5 M HCl, 500 µl distilled water and 10 ml of Parkard Scintillator 299™ were also added. The radioactivity of the samples was determined by liquid scintillation counting. A LKB Wallac 1211 Rackbeta Liquid Scintillation Counter was programmed to count each sample for 10 minutes and corrected for quenching using a calibration curve constructed for the external standard in the presence of ^{3}H. The LKB package was used to convert the results into dpm. The results obtained were corrected for the recovery of oleic acid into the top phase of the extraction, which had been determined to be 82% (Dodds et al., 1987). The results were then converted into activity of LPL expressed as nmoles oleic acid released per minute per mg of postheparin plasma protein.

Statistical analyses

The data were analysed by analysis of variance (ANOVA) using the general linear model (PROC GLM, SAS, 1988). A least significant difference (LSD) was used to compare the differences between means. Correlation analyses were carried out using Pearson's correlation method. Multiple regression analysis was performed using Minitab (Release 11, 1997) statistical software program.

RESULTS AND DISCUSSION

Four initial experiments were performed to determine the appropriate working conditions for the postheparin plasma LPL assays (figure 1). Preincubation of postheparin plasma with SDS (20-50 mM) at 26°C for 45 minutes inhibited hepatic lipase activity (figure 1a). The total VLDL (2 µg/assay) produced maximum stimulation of LPL activity (figure 1b). Quantity of enzyme protein (postheparin plasma) (figure 1c) and increasing incubation time (figure 1d) gave a first order response. The concentration of SDS used routinely was 20 mM, the concentration of postheparin plasma used in subsequent assays was within the established linear range and the incubation period routinely used was 30 minutes.

The results of postheparin plasma LPL activity, proportion of subfraction 2 and backfat thickness for two different genetic lines of pigs are presented in table 2. The proportion of subfraction 2 for LGAH pigs was significantly higher ($p<0.05$) than that of subfraction 2 for VFIL pigs. The postheparin plasma LPL activity, backfat thickness and VLDL lipids, however, were not significantly different ($p>0.05$) between the two lines of pigs.

LPL is found in several extrahepatic tissues and released into the bloodstream by heparin (Brown et al., 1975). The rapid release of enzyme from tissue suggests that heparin interacts strongly with the enzyme in comparison with the cellular site where LPL is bound (Egelrund and Olivecrona, 1972; Bensadoun et al., 1974). The enzyme hydrolyses triacylglycerol in plasma lipoproteins mainly VLDL and

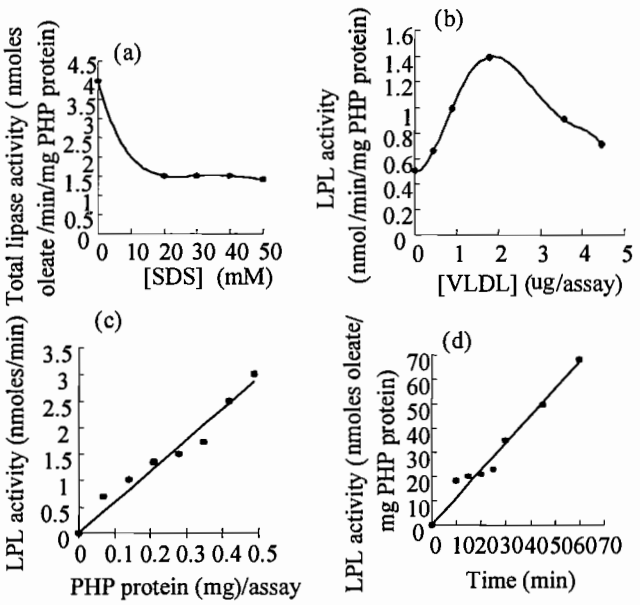

Figure 1. Experiments to determine the appropriate working conditions for the postheparin plasma LPL assays. Assays were performed as described in Material and Methods except that (a) the indicated concentration of SDS (b) the indicated concentration of VLDL protein (c) the indicated concentration of postheparin plasma (d) the indicated incubation time were used in place of the standard conditions. The units of LPL activity are indicated on the Y-axis of the respective graphs.

chylomicrons (Nilsson-Ehle et al., 1980). Many studies show that a reduction in postheparin plasma LPL activity is closely related to hypertriacylglycerolaemia in human patients (Fredrickson et al., 1963; Boberg, 1970). In the experiments reported here, we therefore tested the hypothesis that postheparin plasma LPL activity is higher in pigs with thicker backfat than in those with thinner backfat. The results (table 2), however, show that postheparin plasma LPL activities were not significantly different ($p>0.05$); this finding could be attributed to the backfat thicknesses not being statistically significant ($p>0.05$) in the two lines of pigs. Other studies have shown that genetically obese (fa/fa) Zucker rat (Cleary et al., 1980) and obese human subjects (Taskinen et al., 1980) have elevated cellular levels of adipose tissue LPL.

Lee and Kauffman (1974) reported that LPL activity in subcutaneous swine adipose tissue increased two to three fold between 2 and 24 weeks of age. In another study with obese Minnesota No. 1 swine and lean Hampshire swine, LPL activity was about four times greater in outer subcutaneous adipose tissue of the obese animals (cited from Kris Etherton and Etherton, 1982). The results could be due to the two different breeds of pigs, which were used in their experiments. In contrast, only Landrace pigs were used in our study, which may contribute to the similar results of postheparin plasma LPL activity in the two different genetic lines of pigs.

Correlation analysis (Minitab, 1997) was performed on the results of backfat thickness and proportion of subfraction 2 and postheparin plasma LPL activity. The results show that there were positive correlations between backfat thickness and proportion of subfractions 2 ($R=0.34$, $p=0.14$) and postheparin plasma LPL activity ($R=0.40$, $p=0.24$); however, the correlations were not statistically significant ($p>0.05$). In a previous study (Loh et al., 1997), backfat thicknesses of 80 Landrace pigs were significantly positive correlated with subfraction 2. This was because larger sample size with greater differences of backfat

Table 2. Postheparin plasma lipoprotein lipase activity, proportion of subfraction 2 and backfat thickness for LGAH and VFIL pigs

Parameters		LGAH	VFIL	P
N		8	8	
Postheparin plasma LPL activity (nmoles oleate per min per mg enzyme protein)		1.95±0.56	1.60±0.54	NS
Subfraction 2 (%)		8.57±0.43	6.67±0.32	*
Backfat thickness, mm		10.28±0.43	9.08±0.32	NS
VLDL	Triacylglycerol	110.00±7.10	130.00±16.00	NS
(g/ml)	Total cholesterol	10.00±0.70	10.00±1.30	NS
	Free cholesterol	60.00±0.50	80.00±1.03	NS
	Phospholipid	20.00±1.03	20.00±2.30	NS
	Protein	40.00±4.90	60.00±9.90	NS
	Triacylglycerol:protein	1.04±0.30	1.32±0.46	NS
	Total cholesterol:protein	0.08±0.02	0.10±0.03	NS
	Free cholesterol:protein	0.06±0.02	0.08±0.03	NS
	Phospholipid:protein	0.17±0.04	0.21±0.08	NS

NS, $p>0.05$; * $p<0.05$. The results are presented as mean±SEM.

Table 3. Multiple regression of postheparin plasma LPL activity and proportion of subfraction 2 on backfat thickness

Variables	Constant	LPL	Subfraction 2	R	P
Backfat thickness	6.17	0.53	0.36	0.54	0.11

$n = 16$

thickness used in that study.

A multiple regression analysis (Minitab, 1997) was carried out to examine the effects of the independent variables, proportion of subfraction 2 and postheparin plasma LPL activity on the dependent variable, backfat thickness. The coefficients indicated that the proportion of subfraction 2 and postheparin plasma LPL activity had a positive effect on backfat thickness; however, the results were not statistically significant ($p>0.05$, table 3). Although the correlation between backfat thickness and postheparin plasma LPL activity and proportion of subfraction 2 ($R=0.34$, $p=0.14$; $R=0.40$, $p=0.24$; respectively) and their multiple regression ($R=0.54$, $p=0.11$; table 3) in the two lines of pigs were not statistically significant ($p>0.05$), they still show the positive trends of relationships. It is encouraging that the general trends obtained are consistent with Huttunen et al. (1976), who reported that the activity of human postheparin plasma LPL activity for obese humans was higher than non-obese subjects.

A positive correlation between adipose tissue LPL activity and fat cell size has in fact been reported in animal (Rath et al., 1974) and in human (Taskinen and Nikkilä, 1977). In the present experiment, studies of the size and number of adipocytes were not undertaken. However, it is supposed that obesity is usually accompanied by both hyperplasia and hypertrophy of adipocytes in growing pigs (Kirtland and Gurr, 1979). The postheparin plasma LPL activity and subfraction 2 of thicker backfat pigs is believed to be high rather than low and the apolipoprotein E, which is present in higher quantities in VLDL-subfraction 2 plays an important role for clearing VLDL triacylglycerol into adipose tissue.

In conclusion, LPL activity of pigs can be measured by using postheparin plasma technique without suffering the animals by biopsy or killing them. If the relationships of backfat thickness and VLDL-subfraction 2 and postheparin plasma LPL activity can be established, it suggests that these parameters could be used as indicators in selection programmes. Further experiments need to be conducted by using larger sample size and different breed of pigs with greater differences in backfat thicknesses to confirm these trends.

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