Clearing-Factor Lipase in Muscle and Adipose Tissue of Pigs

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1. Clearing-factor lipase was assayed in acetone–ether-dried powders of heart and adipose tissue of pigs. The enzyme activity in heart was higher than that in adipose tissue. The activity in the outer layer of subcutaneous fat was greater than that in the inner subcutaneous fat and the perirenal fat, which had similar activities. 2. Starvation for 48 h, but not for 24 h, decreased the activity of the heart enzyme. 3. Starvation for 24 h caused a rapid decrease in the activity in all three adipose tissues, but even after 72 h of starvation the activity was still highest in the outer subcutaneous fat. 4. Plasma fatty acid, glucose and insulin concentrations were determined in fed and starved pigs. Starvation decreased the plasma insulin concentration and increased the non-esterified fatty acid concentration.

Clearing-factor lipase has been extensively studied in man and experimental animals (for a review see Robinson, 1970). The enzyme is believed to control the extent to which tissues take up plasma triglycerides (Bezman *et al.*, 1962). During starvation the activity of clearing-factor lipase in rat adipose tissue decreases whereas the activity in heart muscle increases (Hollenberg, 1959, 1960). Such reciprocal changes indicate the physiological importance of this enzyme in regulating the pattern of uptake of the plasma triglyceride fatty acids within the animal. Glucose and insulin are necessary to maintain the clearing-factor lipase activity of rat adipose tissue *in vitro* (Salaman & Robinson, 1966). *In vivo*, glucose can restore the activity in starved animals (Garfinkel *et al.*, 1967) and a requirement for insulin has been shown with alloxan-diabetic rats (Schnatz & Williams, 1963; Brown *et al.*, 1967). However, the enzyme activity in rat heart is increased in diabetes and starvation and decreased by feeding glucose (Borensztajn *et al.*, 1970).

The way in which insulin changes clearing-factor lipase activity in adipose tissue is not completely understood. However insulin decreases the concentration of cyclic AMP in adipose tissue (Butcher *et al.*, 1966) and compounds that stimulate cyclic AMP production decrease clearing-factor lipase activity (Wing & Robinson, 1968). The adipose tissue of the pig, however, has been shown to be insensitive to catecholamines and pituitary peptide hormones *in vitro* (Rudman *et al.*, 1963), many of which function by stimulating adenyl cyclase (Sutherland *et al.*, 1968). Further, lipogenesis from glucose is not stimulated by insulin in pig adipose tissue (O’Hea & Leveille, 1968). In the present study the activity of clearing-factor lipase has been determined in both

heart and adipose tissue of pigs. The effect of starvation on clearing-factor lipase and on the plasma concentrations of insulin, glucose and fatty acids was also measured in an attempt to determine which factors regulate clearing-factor lipase in the pig.

Tissue from three fat depots in the pig was investigated. The presence of different quantities of linoleic acid in different fat depots (Lea *et al.*, 1970) suggested that clearing-factor lipase might be important in this distribution because linoleic acid, an essential fatty acid, is derived solely from the diet.

Materials and Methods

Animals

In early experiments pigs of mixed breed were obtained, but the activity of clearing-factor lipase varied greatly. A few experiments with these pigs have been included but all the large-scale experiments were performed on tissues obtained from pure-bred Large White pigs from a single commercial breeder. The pigs were obtained at weights between 45–55 kg and were kept in loose boxes on straw bedding until slaughtered or starved. They were fed on BOCM-Silcock no. 438 nuts *ad libitum* and allowed free access to water. The animals were slaughtered between 09:15 and 10:15 h and starvation studies were started by the removal of food at 09:15 h. During starvation the pigs were kept in pens with a concrete floor which was covered with sand, some of which they ate. Equal numbers of gilts (young females) and castrated males were used unless otherwise stated. However preliminary results showed no differences between them and the results have been pooled.

Tissue preparations

Pigs were stunned electrically and then bled. A sample of the complete thickness of subcutaneous fat was then removed from the midline of the back in the shoulder region by using a stainless-steel cork-borer (1.6 cm diam.). The carcass was scalded, de-haired and eviscerated and a sample of fat was removed from the perirenal depot just anterior to the right kidney. The fat samples were placed in oxygenated Krebs–Ringer phosphate buffer (pH 7.4) (Krebs, 1933) at 37°C (to facilitate homogenization) in a Thermos flask and taken to the laboratory immediately. The heart was also removed and placed in ice. The time from stunning to the removal of the heart was 15 min. A sample of muscle, free from fat and epimysium, was removed from the tip of the left ventricle and a 3% (w/v) homogenate prepared in 0.025 M-NH₄Cl buffer (pH 8.1, 4°C). The homogenate was used to prepare an acetone–ether-dried powder by the method of Borensztajn *et al.* (1970).

The subcutaneous adipose tissue of pigs is separated by a sheath of connective tissue into an inner and outer layer. The connective tissue was dissected out and the skin removed from the outer layer. Samples (1 g), if possible, were then taken such that each consisted of a section of the whole thickness of each layer. The perirenal adipose tissue was prepared by removing the gross fascia. A 12.5% (w/v) homogenate was prepared from each fat sample in 0.025 M-NH₄Cl buffer (pH 8.1, 37°C) by using a Silverson homogenizer with a micro-tubular head (Silverson Machines Ltd., Chesham, Bucks., U.K.). The homogenate (8.0 ml) was poured into a mixture of acetone (200 ml) and diethyl ether (150 ml) at 0°C; acetone alone was not used because of the low solubility of pig fat. The precipitate was left at room temperature for 1 h, then filtered and washed with ether (200 ml) at room temperature. All acetone–ether-dried powders were stored overnight at 4°C *in vacuo* before assay.

Blood samples were collected at slaughter into plastic centrifuge tubes containing a small quantity of heparin powder. The tubes were capped and centrifuged at approx. 2000g for 15 min at room temperature. The plasma was removed and samples were immediately taken for unesterified fatty acid and glucose analysis. The remainder was stored at –20°C. Serum for clearing-factor lipase assays was obtained from recalcified citrated plasma (Salaman & Robinson, 1966) prepared from pig blood obtained at slaughter.

Clearing-factor lipase assay

The methods of Schotz *et al.* (1970) and Borensztajn *et al.* (1970) as modified by Enser (1972) were used to determine clearing-factor lipase. The assay medium

contained 0.3 vol. of serum for both heart and adipose tissue but 0.375 M-NaCl (0.2 vol.) replaced the 0.154 M-NaCl in the assays of adipose tissue clearing-factor lipase. The acetone–ether-dried powder from 200 mg of heart muscle was homogenized in 20 ml of 0.025 M-NH₄Cl (pH 8.1) and the powder obtained from 1 g of adipose tissue was homogenized in 8.0 ml. A sample (1.25 ml) of each homogenate was shaken with 2.25 ml of glycerol [1-¹⁴C]trioleate–serum at 35°C for 1 h. The release of fatty acids by homogenates of both heart and adipose tissue acetone–ether-dried powders was linear with time for 2 h. The release of fatty acids by acetone–ether-dried powders of heart was proportional to enzyme concentration between 0.5–4.0 mg of fresh tissue equivalent/ml. For adipose tissue the activity was proportional to tissue concentration with acetone–ether-dried powder from 9–90 mg of tissue/ml. Each tissue was assayed in duplicate.

Pigs used for the determination of clearing-factor lipase in heart were not the same as those used for determinations on adipose tissue. Animals under each set of treatments were assayed as a group, but the treatments were changed randomly. For heart clearing-factor lipase each treatment was studied with four groups each containing three to five animals. For studies on adipose tissue three groups of four animals were used for each treatment.

Determination of plasma unesterified fatty acids

Lipid was extracted from 1.0 ml of fresh plasma with 2.5 ml of Dole's extraction mixture (Dole & Meinertz, 1960). For the quantitative removal of phospholipids a portion (1.6 ml) of the final heptane phase was added to 100 mg of silicic acid, followed immediately by 1.0 ml of chloroform (Ho, 1970). These were mixed and left for 10 min, then samples (1.0 ml) were taken for fatty acid determination by the method of Duncombe (1963) modified so that the organic phase floated on the copper reagent. This was accomplished by preparing the copper reagent in a solution saturated with a mixture of sodium and potassium sulphates and by using chloroform–heptane (4:1, v/v) instead of pure chloroform for the organic phase (Ho, 1970).

Determination of plasma glucose

Fresh plasma was deproteinized with Ba(OH)₂–ZnSO₄ and the glucose concentration was determined by the method of Huggett & Nixon (1957).

Determination of plasma insulin

Insulin was determined on samples of plasma that had been stored at –20°C for up to 3 months. Before sampling the plasma was centrifuged at 17000g for 15 min. The method of Hales & Randle (1963) was

used in a kit form supplied by The Radiochemical Centre, Amersham, Bucks., U.K., with human insulin as the standard.

Chemicals

Chemicals were obtained from the same sources and purified as described previously (Enser, 1972).

Glycerol [$1\text{-}^{14}\text{C}$]trioleate was purified by t.l.c. on silica gel G plates developed with hexane–diethyl ether–acetic acid (100:20:2, by vol.). The bands were detected by spraying with 0.2% (w/v) 2,7-dichlorofluorescein in 95% ethanol. The triolein band was scraped into a column and eluted with chloroform. The eluate was concentrated and washed three times with 3.0 ml of 0.01 M-NaOH followed by three washes with distilled water to remove the dichlorofluorescein. Mallinkrodt silicic acid AR, 100 mesh, was obtained from Camlab Ltd., Cambridge CB4 1TH, U.K.

Presentation of results

Clearing-factor lipase activity is expressed as μmol of fatty acid released/h per g of fresh tissue.

Results

Characterization of the enzyme

Clearing-factor lipase is characterized by its requirement for a lipoprotein substrate and by its inhibition by 1.0 M-NaCl (Korn, 1955). The lipoprotein substrate can be prepared by incubating triglyceride emulsions with serum (Enser, 1972). The effect of increasing serum concentration on the lipase of pig heart was similar to that of mouse heart (Enser, 1972) with an optimum serum/triolein emulsion ratio of 0.8; the optimum ratio for pig adipose tissue was 0.6. Assays in the absence of added enzyme showed that the pig serum contained no lipase active under these conditions.

The effects of increasing concentrations of NaCl on the activity of clearing-factor lipase from pig heart and adipose tissue are shown in Fig. 1.

Clearing-factor lipase in heart

The activity of clearing-factor lipase in pigs which had continuous access to food was 24.5 ± 1.5 (18) units/g fresh tissue. Since the cardiac enzyme has been shown to increase during starvation in other species the activity was determined in pigs which had been starved for 24 and 48 h. Starvation for 24 h did not affect the activity, but there was a marked decrease after 48 h (Table 1).

Clearing-factor lipase activity of adipose tissue

The main fat depot in the pig is the subcutaneous adipose tissue. The clearing-factor lipase activity recovered from the outer layer (see the Materials and

Fig. 1. Effect of NaCl on clearing-factor lipase activity

Heart enzyme (●) was from a fed female pig, carcass wt. 50 kg. Adipose tissue enzyme (○) was from a fed castrated male pig, carcass wt. 78.5 kg. Solid NaCl was added to the assay flasks and dissolved in the incubation medium before the addition of enzyme.

Table 1. Effect of starvation on clearing-factor lipase activity of pig heart

For experimental details see the text. The results are means $\pm$ S.E.M.; 18 animals were used for each treatment.

	Period of starvation (h) ...	0	24	48
Clearing-factor lipase activity (μmol of fatty acid released/h per g of fresh tissue)		24.2 ± 1.5	23.4 ± 1.2	15.5 ± 1.7
Carcass wt. of animals (kg)		61.8 ± 2.6	54.5 ± 0.8	59.1 ± 0.8
Sex of pigs		10 ♀, 8 ♂	8 ♀, 10 ♂	10 ♀, 8 ♂

Methods section) in fed pigs was 17.4 ± 1.02 (12) units/g of fresh tissue and was 46% higher than that in the inner layer. However, there was no significant difference between the activity in the inner subcutaneous fat, 11.9 ± 1.4 (12) and the perirenal fat, 10.4 ± 1.3 (12) (Fig. 2). The quantity of acetone-ether-dried powder obtained (mg/g of adipose tissue) was: outer subcutaneous 30.5 ± 1.5 ; inner subcutaneous 24.3 ± 2.1 ; perirenal 16.7 ± 1.0 .

Effect of starvation on the clearing-factor lipase activity of adipose tissue

The activity of clearing-factor lipase decreased rapidly in all three fat depots studied (Fig. 2); after starvation for 48 h the activity of the enzyme had decreased by more than 90% in all three depots. Between 48 and 72 h there were further small decreases in activity but that in the outer subcutaneous layer remained higher than that in the inner layer.

Plasma concentrations of glucose, insulin and fatty acids

Glucose and insulin were assayed in the plasma of fed and starved pigs to determine whether any changes in their concentrations were correlated with changes in the activity of clearing-factor lipase. In fed pigs the plasma glucose concentration was 70 ± 2.6 (12) mg/100 ml of plasma. After 24 h of starvation it decreased by 18%, but during further starvation it increased until after 72 h it was very close to the concentration in fed pigs (Table 2).

The concentration of insulin in plasma of fed pigs was somewhat variable with a mean value of 48 ± 7.4 (12) μ units/ml. It decreased by 65% after 24 h of starvation and remained at this value during subsequent starvation up to 72 h (Table 2).

The lipolytic response of pig adipose tissue to star-

vation was investigated by following changes in the concentration of unesterified fatty acids in plasma. In the fed pig the concentration was 0.168 ± 0.064 (12) mequiv./litre of plasma; it increased during starvation

Fig. 2. *Effect of starvation on the clearing-factor lipase activity of subcutaneous and perirenal adipose tissue from pigs*

The activity was assayed in duplicate in acetone-ether-dried powders from individual pigs. Twelve pigs were used at each stage and the activity in all three fat depots was assayed in each pig. ●, Outer subcutaneous adipose tissue; ○, inner subcutaneous adipose tissue; ■, perirenal adipose tissue. Bars represent the s.e.m. Where it is not shown it was smaller than the symbol. For the details of the weights of the pigs and weight loss during starvation see Table 2.

Table 2. *Effect of starvation on the plasma concentrations of glucose and insulin*

For experimental details see the text. Results are means $\pm$ s.e.m. Twelve animals were used for each treatment (six gilts and six castrated males)

	Period of starvation (h) ...	0	24	48	72
Plasma glucose concentration (mg/100 ml)		70.0 ± 2.6	57.3 ± 2.4	65.9 ± 1.6	69.4 ± 1.8
Plasma insulin concentration (μ units/ml)		48.0 ± 7.4	16.8 ± 1.9	14.8 ± 1.2	17.7 ± 1.4
Wt. of pigs before starvation (kg)		78.7 ± 1.1	72.0 ± 1.6	69.1 ± 1.2	73.4 ± 1.7
Wt. of pigs after starvation (kg)		—	69.0 ± 1.6	65.0 ± 1.1	68.2 ± 1.7
% wt. loss during starvation		—	4.2	5.9	7.1

up to 48 h (3–4-fold increase) and then decreased slightly up to 72 h.

Discussion

Clearing-factor lipase in pig heart muscle and adipose tissue has the usual characteristic requirement for serum and is inhibited by 1 M-NaCl. Activation of the rat enzyme by low concentrations of NaCl has recently been reported by LaRosa *et al.* (1972) by using acetone–ether-dried powder from various tissues.

The activity of clearing-factor lipase in pig heart is approximately one-twelfth of that in mice (De Gasquet & Pequignot, 1972; Enser, 1972). This low activity and the absence of an increase during starvation may indicate that the triglycerides of plasma lipoproteins are not an important source of energy for heart in the pig, especially during starvation.

The activity of clearing-factor lipase in acetone–ether-dried powders of pig adipose tissue is similar to that found in the epididymal fat-pads of mice. The decrease in activity during starvation indicates that the enzyme in pigs is as susceptible as that in other species to physiological changes. This decrease in adipose tissue clearing-factor lipase activity is correlated with the plasma insulin concentration and inversely correlated with the plasma fatty acids. The effect of insulin on clearing-factor lipase activity in adipose tissue of rats *in vitro* and *in vivo* is well established (Wing *et al.*, 1966; Meng & Goldfarb, 1959; Schnatz & Williams, 1963), whereas Wing & Robinson (1968) observed that clearing-factor lipase activity could be decreased in the absence of an increase in the tissue fatty acid concentration. The increase in pig plasma fatty acids during starvation in the present study is similar to that found by Hertelendy *et al.* (1970), but lower than the changes reported by Cunningham & Friend (1965), who used a less specific assay. Both the decrease in adipose tissue clearing-factor lipase activity and the increase in plasma fatty acids could be produced by a decrease in plasma insulin concentrations. The low concentration of glucose in the plasma of fed pigs in this study was surprising. Machlin *et al.* (1968) found 148 mg/100 ml of glucose in plasma from venous blood samples of fed pigs and 90 mg/100 ml in pigs starved for 24 h.

We did not find a correlation between the lipase activities and the linoleate contents (Lea *et al.*, 1970) of the different adipose tissues; it would however be premature to conclude that clearing-factor lipase is not important in regulating the fatty acid composition of the fat depots.

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