Influence of adipocyte isolation by collagenase on phosphodiesterase activity and lipolysis in man

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Abstract The maximum phosphodiesterase activity (V_{max}) with low and high K_m was, respectively, 10- and 3-times greater in tissue fragments than in collagenase-isolated adipocytes obtained from subcutaneous fat layers in man. The exposure of such tissue fragments to collagenase of various origins in order to isolate the fat cells resulted in a 60–70% inhibition of phosphodiesterase (PDE) activity. Noradrenaline- and isopropyl noradrenaline-induced rates of lipolysis were more rapid in the isolated fat cells than in the tissue fragments. The sensitivity to catecholamines, however, was the same for the two tissue preparations. Nor did they differ in respect to the effect of theophylline, a PDE inhibitor, on the rate of lipolysis. The time curve for cyclic AMP accumulation was significantly higher in the isolated adipocytes than in tissue fragments in the presence of isopropyl noradrenaline. It is concluded that the greater lipolytic response of collagenase-isolated adipocytes than of tissue fragments to catecholamines may be attributed, at least in some measure, to the higher concentration of cyclic AMP resulting from a decrease in PDE activity.—**Engfeldt, P., P. Arner, and J. Östman.** Influence of adipocyte isolation by collagenase on phosphodiesterase activity and lipolysis in man. *J. Lipid Res.* 1980. **21:** 443–448.

Supplementary key words human adipose tissue · cyclic AMP

The use of fat cells isolated with collagenase by the method of Rodbell (1) rather than fragments of human adipose tissue avoids any contribution of cyclic AMP (cAMP) (2) from the stromal cells. A drawback of the method, however, is the substantial loss of fat cells during the isolation procedure, especially when human material is used (3). Another disadvantage is the possible selection of small adipocytes, such as has been reported in the rat (4).

The present study was promoted by the discovery of elevated levels of cAMP in adipose tissue of patients with diabetes mellitus (5), patients with obesity who had been submitted to therapeutic starvation (5), and patients with hyperthyroidism (6). The main object was to compare the activity of phosphodiesterase (PDE) in fragments of adipose tissue with that in isolated fat cells. The two tissue preparations were found to display a considerable difference in PDE activity, and the possible relevance of this difference for the lipolytic response to catecholamines was accordingly investigated.

MATERIAL AND METHODS

Subcutaneous adipose tissue was obtained at operations from patients undergoing elective cholecystectomy. They had fasted overnight. None had jaundice, any metabolic disorder, or malignant disease. No attempt was made to select the patients on the basis of age, sex, or body-weight. General anaesthesia was induced with a short-acting barbiturate and maintained with halothane. The specimen of adipose tissue was taken at the start of the operation, and segments and isolated fat cells were prepared by the methods described below.

Isolation of fat cells

The adipose tissue specimen was cut into fragments, each weighing about 10 mg. Fat cells were isolated from the stroma by incubation with 5 mg of various types of collagenase for 60 min in 3 ml of Krebs-Ringer bicarbonate (KRB) buffer, containing 40 mg of bovine serum albumin per ml unless otherwise stated, by the method of Rodbell (1), as modified by Smith, Sjöström, and Björntorp (7). This concentration of collagenase is necessary for effective isolation. The adipocytes were washed three times in pure KRB buffer. The yield was about 10^5 isolated adipocytes from 200 mg wet weight of tissue.

Preparation of tissue extract for the PDE assay

Three hundred milligrams of adipose tissue was incubated in 5 ml of KRB buffer without collagenase for 60 min and at 37°C, unless otherwise stated. The fragments were homogenized in glass with 100 mM Tris buffer (pH 7.5) containing 3 mM Mg^{2+}. The

Abbreviations: PDE, phosphodiesterase; cAMP, cyclic AMP; KRB, Krebs-Ringer bicarbonate.

homogenate was sonicated for 10 sec, and then centrifuged at 2000 *g* (4°C) for 10 min. The liquid phase was removed with a syringe for the PDE assay, and the lipids were extracted by the method of Dole and Meinertz (8) for determination of the lipid weight. The protein content was estimated by the method of Lowry and co-workers (9). In the extraction procedure for the adipocytes, about 10^6 cells were resuspended in Tris buffer. The procedure was then the same as that for the tissue fragments, except that the homogenization step was omitted. The PDE activity was expressed in terms of the lipid weight or the amount of protein present. The former was considered to be the more reliable because of possible contamination with stromal protein and albumin in the intact adipose tissue preparations.

PDE activity determination

The PDE activity was assayed by the method of Pöch (10). The substrate used was ^{32}P-labeled cAMP with the concentration series 0.0125, 0.025, 0.05, 0.1, 0.2, 6.25, 12.5, 25 and 50 μM, in Tris buffer containing 3 mM Mg^{2+}. The reaction was initiated by adding ^{32}P-labeled cyclic AMP and terminated with 0.15 M $ZnSO_4$, when 10–20% of the cAMP had been degraded (after 3–120 min). To precipitate other nucleotides than ^{32}P-labeled cAMP, 0.15 M $Ba(OH)_2$ was added. By incubating at least four aliquots of the sample with the same concentration of cAMP for four different periods, the initial speed of degradation could be calculated using computerized curve adjustment. Lineweaver–Burk (11) plots of the PDE activities showed that each curve could be approximated to two straight lines. This indicated the presence of two forms of the enzyme, one with a low and the other with a high K_m value. The coefficient of variation for the method was about 10%.

Measurement of rate of lipolysis

Fragments of adipose tissue each weighing about 100 mg, or about 10^5 isolated adipocytes, were preincubated for 30 min in 1 ml of KRB buffer (pH 7.4 and 37°C) containing 40 mg of bovine serum albumin per ml. Segments or isolated cells were then incubated in 1 ml of fresh medium of the same type for 2 hr. Two aliquots of the medium (each of 0.1 ml) were taken for glycerol estimation by the method of Wieland (12) as modified by Chernick (13). Each incubation was run in triplicate or quadruplicate. Further details have been given elsewhere (14).

Assay of intracellular cAMP

The intracellular cAMP concentration was determined in adipose tissue fragments and isolated adipocytes preincubated for 30 min in KRB-buffer (pH 7.4 and 37°C) containing 10 mg of bovine serum albumin per ml and with or without 10 mM of theophylline per liter. The incubation was carried out for different periods of time, using 1 ml of buffer to 100 mg of adipose tissue, or 3×10^5 fat cells.

Cyclic AMP was determined by a modification of the protein-binding method of Gilman (15); it has been described in detail elsewhere (16).

Chemicals

Bovine serum albumin, fraction V, was purchased from Armour Pharmaceutical Company, Eastborne, England. The various forms of collagenase prepared from *Clostridium histolyticum* were of Sigma type I (Lot nos. 87C-0364 and 126C-0435), Worthington (Lot no. CLS96F), and Boehringer-Mannheim (Lot no. 1198426). Trypsin was of Sigma type III (Bovine pancreas, Lot no. 37C-04231). Trypsin inhibitor (Hen egg white, Lot no. 1536410), cAMP-binding protein and [^{3}H]adenosine-3′,5′-monophosphate (cyclic ammonium salt with a specific activity of 15 mCi/mmol) were all from Boehringer-Mannheim. Beef heart PDE was from Sigma. ^{32}P-labeled adenosine-3′,5′-monophosphate (triethylammonium salt with a specific activity of 2–20 Ci/mmol) was from New England Nuclear Corp. Noradrenaline bitartrate was from Astra, Sweden, isopropyl noradrenaline hydrochloride from Winthrop, England, and theophylline from ACO, Sweden.

Determination of fat-cell size

The fat-cell diameter was determined as described by Sjöström, Björntorp, and Vrána (17). One hundred cells were measured and the mean cellular triglyceride content was calculated from the mean diameter. The standard deviation of the diameter was calculated from a formula evolved by Hirsch and Gallian (18). In separate experiments the mean fat-cell size was determined after isolation with collagenase. No statistically significant difference in mean diameter or size distribution of the fat cells was observed between fragments of adipose tissue and isolated fat cells.

Statistical analysis

The values given are the mean and the standard error of the mean. The significance of the differences was calculated by Student's paired or unpaired *t* test, as described by Snedecor (19).

RESULTS

The results of the assays of phosphodiesterase activity in the adipose tissue fragments and the isolated

TABLE 1. Comparison of PDE activity in isolated fat cells and fragments of human subcutaneous adipose tissue from seven patients

	Low K_m PDE		High K_m PDE	
	K_m	V_{max}	K_m	V_{max}
	(μM)	*(pmol/min/g lipid)*	*(μM)*	*(pmol/min/g lipid)*
Isolated fat cells	0.16 ± 0.04	12 ± 3	56 ± 7	2010 ± 325
Adipose tissue fragments	0.45 ± 0.10	131 ± 26	68 ± 6	6068 ± 335
	$P < 0.05$	$P < 0.01$	$P < 0.05$	$P < 0.001$

Fragments and isolated fat cells were prepared from adipose tissue of each donor, (buffers without albumin were used). The phosphodiesterase (PDE) activity was determined at a range of cAMP concentrations. K_m and V_{max} were calculated according to Lineweaver and Burk.

Values are mean ± standard error of mean. *P* denotes the statistical significant difference obtained with Student's paired *t* test.

fat cells are presented in **Table 1** and representative kinetic data from one experiment are shown in **Fig. 1.** Expressed in terms of lipid weight, the peak of PDE activity recorded with low and high K_m was 10- and 3-times greater in the tissue fragments than in the isolated fat cells ($P < 0.01$ and $P < 0.001$, respectively). The K_m of the two enzyme activities was also significantly higher in the tissue fragments than in the isolated fat cells ($P < 0.05$). When the PDE activity was expressed in terms of the amount of protein, for both forms of PDE, the V_{max} and the K_m were also higher in the adipose tissue fragments than in the isolated adipocytes.

The tissue fragments experiments showed a positive linear correlation between the mean fat-cell volume and the V_{max} of the low K_m PDE activity ($r = 0.67$, $P < 0.01$, n = 18) (**Fig. 2**). Similar results were obtained with high K_m ($r = 0.73$, $P < 0.05$, n = 10) (uncharted experiments).

Exposure of adipose tissue fragments to collagenase (Sigma type I) at a concentration of 5 mg in 3 ml of buffer resulted in a decrease of about 70% of V_{max} of both the low and high K_m PDE after 15 min. After that time no further changes occurred in the PDE activity. At 60 min the PDE activity was similar in isolated adipocytes and in isolated adipocytes plus stroma. The PDE activity in isolated adipocytes did not change even if they were washed several (six) times. Even at a collagenase concentration of 1.5 mg in 3 ml the PDE activity was significantly reduced by more than 50%.

Fifteen-minute incubation of tissue fragments with collagenase (5 mg/3 ml) of various forms (Sigma type I, Worthington and Boehringer) significantly decreased the V_{max} of both the low (**Fig. 3**) and the high (data not shown) K_m PDE by 60–70%. Repeated washes (ten times) of tissue fragments with buffer without collagenase did not increase the PDE activity.

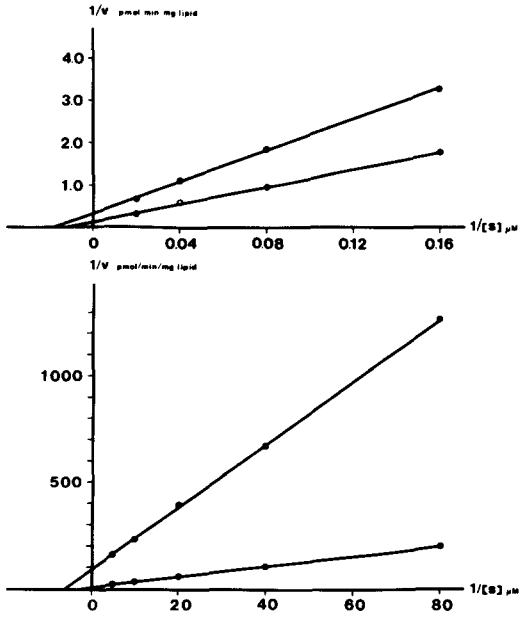

Fig. 1. Double reciprocal plot of kinetic data for phosphodiesterase activity from isolated fat cells (closed circles) and adipose tissue fragments (open circles) in one typical experiment. In the upper panel the cAMP concentration varied from 6.25 to 50 μM. (Isolated fat cells: K_m = 59 μM, V_{max} = 3.226 pmol/min/mg lipid. Adipose tissue fragments: K_m = 80 μM, V_{max} = 7.874 pmol/min/mg lipid). In the lower panel the cAMP concentration varied from 0.0125 to 0.2 μM. (Isolated fat cells: K_m = 0.18 μM, V_{max} = 0.012 pmol/min/mg lipid. Adipose tissue fragments: K_m = 0.39, V_{max} = 0.160 pmol/min/mg lipid).

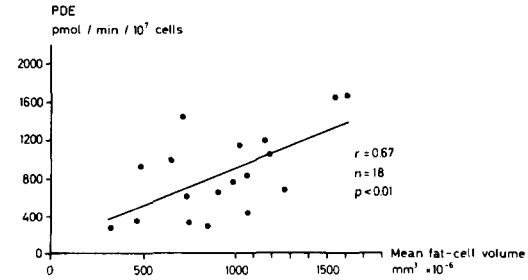

Fig. 2. Relationship between mean fat-cell volume and phosphodiesterase (PDE) activity (V_{max} of the low K_m form) in human adipose tissue fragments.

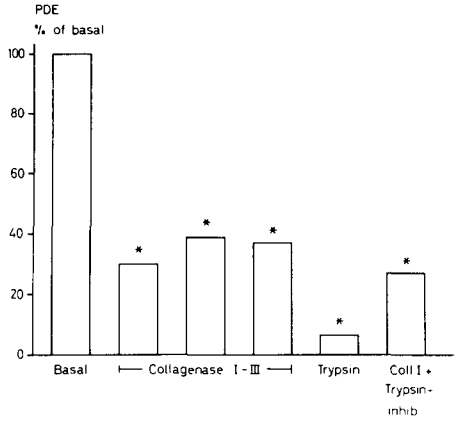

Fig. 3. Effect of trypsin, trypsin inhibitor and various types of collagenase on phosphodiesterase (PDE) activity (V_{max} of the low K_m form) in human adipose tissue. Tissue fragments, each weighing about 10 mg, were incubated for 15 min in buffer with or without (basal) collagenase (I: Sigma type I, II: Worthington, III: Boehringer), trypsin (1 mg/ml) or trypsin inhibitor (1 mg/3 ml). *P* denotes the statistical difference between basal and enzyme-induced values using Student's unpaired *t* test (* denotes $P < 0.001$). Each experiment was run in quadruplicate.

A pronounced inhibition of PDE activity was also obtained with trypsin. The presence of trypsin inhibitor did not interfere with the effect of collagenase on the PDE activity.

Addition of collagenase (1 mg/ml) to an homogenate from human adipose tissue resulted in a decrease of the V_{max} of both the low and the high K_m PDE with 80–90%. In a separate set of experiments, the addition of collagenase (Sigma type I) at a concentration of only 0.05 mg/ml reduced the PDE activity of purified beef heart phosphodiesterase solution by about 50%.

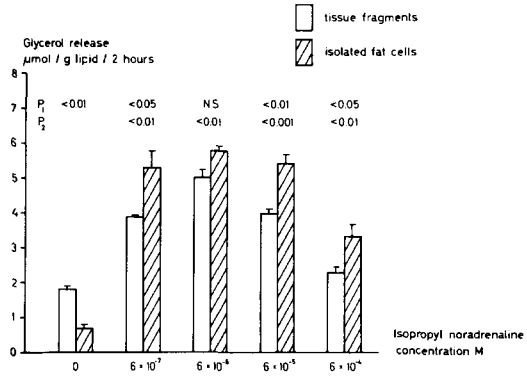

Fig. 4. Effect of graded doses of isopropyl noradrenaline on rate of lipolysis in isolated fat cells and fragments of human adipose tissue. Isopropyl noradrenaline was added to the buffer at concentrations up to 0.6 mM and the glycerol release to the medium was determined after 2 hr of incubation. P_1 and P_2 denote the statistical difference in the glycerol release in isolated fat cells and fragments, (Student's unpaired *t* test). P_1 relates to the total glycerol release, and P_2 the isopropyl noradrenaline-induced glycerol release above baseline. NS = not significant. Each experiment was run in triplicate.

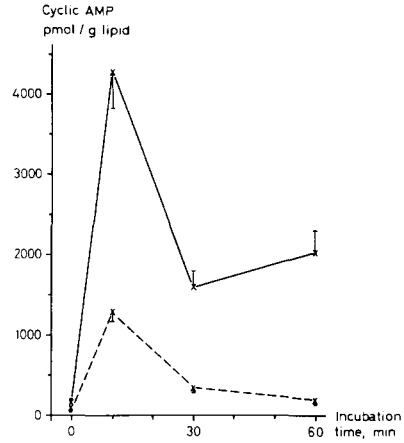

Fig. 5. Time course for cAMP levels in isolated fat cells (solid lines) and fragments (broken lines) of human adipose tissue exposed to 6 μM of isopropyl noradrenaline. Intracellular cAMP was determined after incubation for various periods. The levels of cAMP were in every point significantly higher (0 min, $P < 0.05$; 10 min, $P < 0.001$; 30 min, $P < 0.001$; 60 min, $P < 0.01$) in isolated fat cells than in fragments. *n* = four or five incubations.

The basal rate of lipolysis in the isolated fat cells was lower than in the tissue fragments (**Fig. 4**) but the lipolytic response to isopropyl noradrenaline at all concentrations (6×10^{-7}–6×10^{-4} M) was greater. The sensitivity to isopropyl noradrenaline was the same for the two tissue preparations, as is seen from the dose-response curve in Fig. 4; the same applies to the sensitivity to noradrenaline (results not presented). On the other hand, the stimulatory effect of theophylline at a concentration known to almost completely inhibit the PDE activity (10 mM) was of the same order of magnitude in the two tissue preparations.

The time curve for cAMP accumulation in the adipocytes was significantly higher than for fragments in the case of exposure to isopropyl noradrenaline (6×10^{-6} M) (**Fig. 5**). If adipocytes and fragments were exposed to isopropyl noradrenaline (6×10^{-6} M) together with theophylline (10 mM) the time curves for cAMP accumulation were similar (data not shown).

DISCUSSION

In both preparations of human adipose tissue, namely, fragments and isolated fat cells, analysis by the Lineweaver-Burk plot revealed the existence of two PDE activities with different K_m, low and high as reported earlier by Solomon (20). In the present study, negligible levels of PDE activity were detected in stromal cells obtained by the isolation procedure. Further evidence that these cells do not contribute to PDE activity is the observed strong correlation between mean fat-cell size and PDE activity in adipose

tissue fragments; this gave a regression line with a small intercept on the *y* axis.

The considerably lower V_{max} of the low and the high K_m PDE in isolated fat cells than in tissue fragments is probably due to the collagenase. For all the types and batches of collagenase that were tested there was a rapid and notable decrease in the PDE activity. The addition of trypsin inhibitor did not prevent the collagenase-induced decrease in PDE activity. Whereas collagenase-isolated fat cells in the present study exhibited a greater response to catecholamines, it has been shown that trypsin inhibited the lipolytic response to noradrenaline and low concentrations of theophylline in human adipocytes (21, 22).

It is reasonable to suppose that the more pronounced lipolytic response to isopropyl noradrenaline observed for the isolated fat cells than for the tissue fragments is due, at least in part, to the difference in the level of PDE activity of these two tissue preparations. Support for this supposition is that the cAMP accumulation in isolated adipocytes was significantly higher than in tissue fragments after exposure to isopropyl noradrenaline, and in the similarities between the preparations as regards both cAMP accumulation and the rate of lipolysis in the presence of the PDE inhibitor, theophylline. In other words, isopropyl noradrenaline may induce a similar rate of cAMP production in the two tissue preparations, whereas the breakdown of cAMP is probably less marked in the isolated adipocytes exposed to collagenase.

The present findings are in accordance with results reported from rat adipocytes. Rodbell (23) has reported that the lipolytic response to adrenaline was higher in isolated fat cells than in tissue fragments. Fain (24) has found increased lipolytic response to noradrenaline in isolated adipocytes. Consistent with these results is Schimmel's finding (25), also in the rat, that the cAMP accumulation in the presence of adrenaline and theophylline, together, was considerably greater in isolated fat cells than in adipose tissue segments.

It has been demonstrated that the plasma membrane of the chicken embryo fibroblasts contains cAMP-specific PDE, and that the kinetic behaviour of this enzyme is modified by trypsin (26); the K_m of the high affinity site was increased and the V_{max} of both sites were decreased. A similar effect was obtained in intact fibroblasts. Although the effect of trypsin in that study is somewhat different than that of collagenase observed in the present study, it is conceivable that both effects of these agents are possible because the enzyme is located on the surface of the cell. It would appear from the present study that results furnished by human fat cells isolated by collagenase should be interpreted with caution.

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