Insulin-sensitive phosphodiesterase and insulin receptor binding in fat cells from spontaneously obese rats

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Summary. The effects of insulin on insulin-sensitive phosphodiesterase were investigated in fat cells from rats aged 4, 8 and 16 weeks. The enzyme activities in rats aged 4 and 8 weeks higher at 0.1–30 nmol/l insulin concentrations than in rats aged 16 weeks, and half-maximum stimulations were obtained at 0.08 nmol/l in rats aged 4 weeks, at 0.15 nmol/l in rats aged 8 weeks and at 0.22 nmol/l in rats aged 16 weeks. Specific binding of insulin in fat cells from rats aged 4, 8 and 16 weeks was 3.3%, 5.0% and 11.6%/2×10^5 cells, respectively.

Scatchard analysis indicated that increased insulin binding in fat cells from rats aged 16 weeks was due mainly to an increase of binding affinity. These results suggest that impairment of the phosphodiesterase activation system in fat cells from spontaneously obese rats is predominantly due to post-receptor defects.

Key words: Phosphodiesterase, insulin receptor, rat fat cell, spontaneous obesity, post-receptor defects.

Of all the insulin resistant states, the most closely related to the aetiology of non-insulin-dependent diabetes is obesity [1]. Obesity in man and laboratory animals is commonly associated with glucose intolerance, hyperinsulinaemia and resistance to endogenous and exogenous insulin [2]. Insulin resistance at the cellular level may be caused by alterations in insulin receptors or by multiple steps of insulin action beyond insulin receptor binding [3].

Insulin stimulates the membrane-bound low Km cyclic AMP phosphodiesterase, in the case of incubation with intact fat cells [4–6], hepatocytes [4, 7] and muscle [4, 8]. This enzyme seems to be associated with the endoplasmic reticulum [6]. Since the action of insulin on the phosphodiesterase activation system is an acute event similar to that seen in the glucose transport system [5], phosphodiesterase activity should be a useful monitor for changes in insulin action.

Recently, we found that sulphonylurea [9] and concanavalin A [10] activated phosphodiesterase and there was a good correlation between insulin binding and its enzymatic activation in fat cells from streptozotocin-diabetic [11] and adrenalectomized rats [12]. As there are few reports on the relationship between obesity and phosphodiesterase, the present study was undertaken to examine the possible changes seen in the spontaneously obese rat.

Material and methods

Materials

Porcine monocomponent insulin was a gift from Shimizu Pharmaceuticals, Shizuoka, Japan; ^{125}I-monoiodinated insulin (sp. act. 100 μCi/μg) was obtained from New England Nuclear Corporation, Boston, Mass., USA; (8-^{3}H)-cyclic AMP was purchased from Amersham International, Amersham, Bucks, UK; AG1-X2 was from Bio-Rad, Richmond, Calif. USA; crude bovine serum albumin (fraction V; Lot U-73710) was from Armour Pharmaceuticals, Chicago, Ill., USA; crude bacterial collagenase (Lot 108C-0062), snake venom, trypsin and soybean trypsin inhibitor were from Sigma Chemicals, St. Louis, Miss., USA. Other chemicals were either of reagent grade or the best grade available.

Animals

Male Sprague-Dawley rats were maintained at 24 °C under conditions of controlled lighting with free access to standard laboratory chow and tap water from birth to age 4, 8 and 16 weeks (their final weight was approximately 110 g, 240 g and 430 g, respectively).

Methods

Fat cells from epididymal adipose tissue were prepared by the collagenase method [13]. Freshly prepared cells were incubated with various concentrations of insulin for 10 min at 37 °C in Krebs-Henseleit Hepes buffer (pH 7.4), containing 20 mg/ml for fraction V albumin (1–2.4 × 10^6 fat cells in 5 ml incubation volumes). The treated cells were washed twice by centrifugation at 180 g with 0.25 mol/l sucrose/

Table 1. Characteristics of the three groups of rats

Rat age (weeks)	Body weight (g)	Fat tissue weight (g)	Plasma glucose (mmol/l)	Plasma insulin (mU/l)	Fat cell diameter (μm)	Lee index[a]
4 (n=28)	108 ± 16	0.39 ± 0.1	8.8 ± 0.6	36.1 ± 9	42 ± 10	306 ± 10
8 (n=28)	238 ± 27[b]	2.0 ± 0.5[b]	10 ± 1.4[c]	63.6 ± 24[b]	62 ± 13[b]	318 ± 7[b]
16 (n=28)	430 ± 38[b]	7.1 ± 1.9[b]	11 ± 0.9	84.1 ± 18[d]	88 ± 14[b]	327 ± 6[b]

Results expressed as mean ± SD. [a] Lee index $= \dfrac{\sqrt[3]{\text{Body weight (g)}}}{\text{Naso-anal length (cm)}} \times 10^3$ [18]. [b] $p < 0.001$; [c] $p < 0.025$ group 4 weeks versus group 8 weeks or group 8 weeks versus group 16 weeks; [d] $p < 0.05$ group 8 weeks versus group 16 weeks
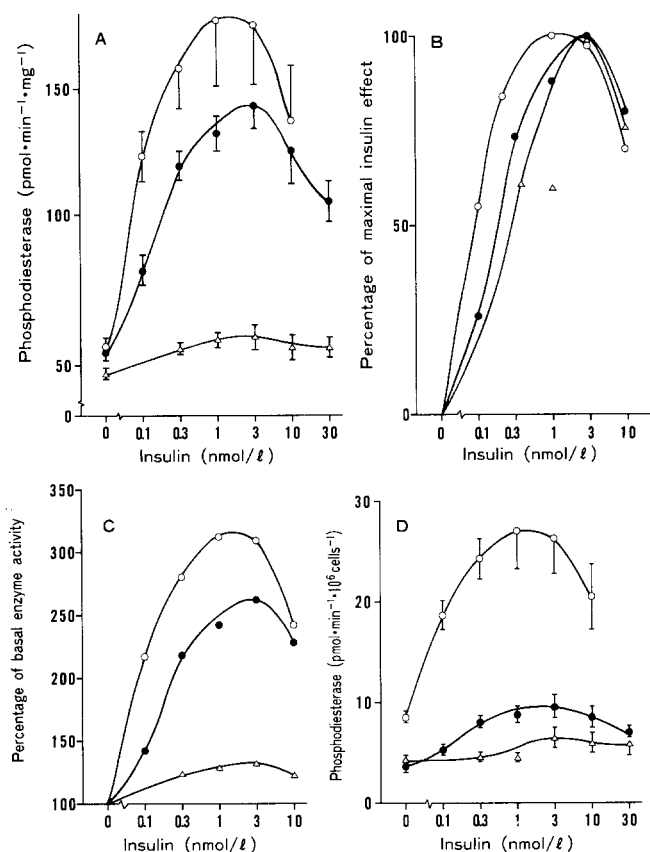

Fig. 1. A Effects of increased insulin concentrations on the phosphodiesterase activities in fat cells from rats aged 4 (O), 8 (●) and 16 (△) weeks. Fat cells were exposed to the indicated concentrations of insulin for 10 min. The enzyme activities were determined in fraction P-2. Each symbol and bar show mean ± SEM (n=6). **B** The data are plotted as a percentage of the maximal insulin effect, calculated by dividing the increment of the enzyme activity at the indicated insulin concentration by the maximal increment (insulin concentration is 1 nmol/l in fat cells from rat aged 4 weeks, 3 nmol/l in fat cells from rats aged 8 and 16 weeks). **C** The data are plotted as a percentage of the basal enzyme activities, calculated by dividing the increment of the enzyme activity at the indicated insulin concentration by the basal increment. **D** The data are shown per 10⁶ cells

10 mmol/l Tes (N-tris[hydroxymethyl] methyl-2-aminoethanesulphonic acid) at 15 °C and homogenized in the buffer. A crude microsomal fraction (fraction P-2), prepared by differential centrifugation as described previously [14], was suspended in sucrose (0.25 mol/l) containing Tes buffer (10 mmol/l pH 7.5) at 0 °C.

Phosphodiesterase in fraction P-2 was assayed as described by

Kono et al. [6]. Briefly, the enzyme was incubated with ³H-cyclic AMP (0.1 μmol/l) for 5 min (4–9 μg protein of fraction P-2 in 250 μl incubation volume), the ³H-AMP formed was degraded by 5′-nucleotidase from snake venom to ³H-adenosine; and the latter was isolated by column chromatography on AG1-X2. For trypsin treatment of phosphodiesterase, an aliquot of fraction P-2 was incubated with trypsin (10 μg/ml) for 10 min at 0 °C (the phosphodiesterase was maximally activated at this concentration of trypsin) and then mixed with soybean trypsin inhibitor (20 μg/ml) [14]. Protein was assayed by the method of Lowry et al. [15] using crystalline bovine serum albumin as the standard.

Isolated fat cells were suspended in Krebs-Henseleit Hepes buffer (pH 7.4), containing ¹²⁵I-insulin and unlabelled insulin in plastic test tubes (1.5–3 × 10⁵ fat cells per 250 μl incubation volume) in a shaking water bath. Optimal steady-state binding conditions were achieved at 24 °C during 60–120 min of incubation. The incubations were terminated after 60 min, as described by Gammeltoft and Gliemann [16], by removing 200 μl aliquots from the cell suspension and rapidly centrifuging at 10000 g in plastic microcentrifuge tubes containing dinonylphthalate (100 μl). The tops of the tubes were cut off and the sediments were counted using a gamma-counter (Wallac Oy, Turku, Finland). More than 95% radioactivity of ¹²⁵I-insulin was precipitated by 10% trichloroacetic acid. All data were corrected for non-specific binding (2–20% of the total amount bound), which was the amount of radioactivity remaining in the bound fractions in the presence of unlabelled insulin (28 μmol/l). After staining with osmium tetraoxide, fat cell size and counts were determined by a microscopic method [17].

Plasma glucose was determined using a kit (Blood Sugar GOD. Perid Test, Boehringer Mannheim, Mannheim, FRG). Plasma insulin was measured by radioimmunoassay using a kit (Phadebas Insulin Test, Pharmacia Diagnostios, Uppsala, Sweden).

Statistical analysis

Results are expressed as mean ± SD or mean ± SEM. Statistical comparisons were carried out using umpaired Student's t-test.

Results

Body weight and epididymal fat tissue weight increased significantly up to 16 weeks of age ($p < 0.001$, Table 1). The mean blood glucose level tended to increase, with a significant difference between rats aged 4 and 8 weeks ($p < 0.025$), but not between rats aged 8 and 16 weeks. Mean plasma insulin levels and fat cell diameter of rats aged 16 weeks were greater than those of rats aged 4 and 8 weeks ($p < 0.05$ to $p < 0.001$). Lee index, which was utilized to assess the degree of obesity, was also significantly increased up to 16 weeks of age ($p < 0.001$) [18].
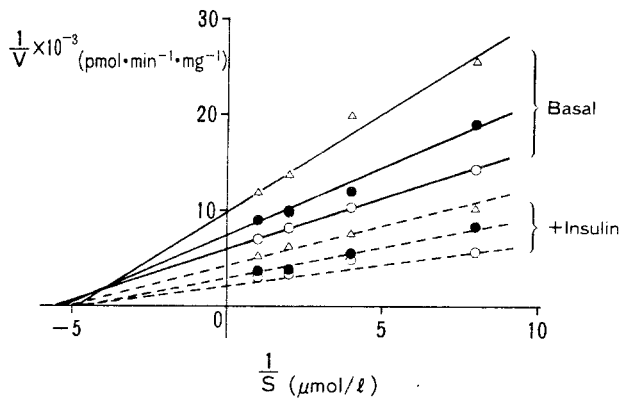
Fig.2. Lineweaver-Burk plots for fat cells from rats aged 4 (○), 8 (●) and 16 (△) weeks (n=3). The calculated apparent K_m and V_{max} values (mean ± SEM) are shown in the text

Table 2. Phosphodiesterase activity after mild trypsin treatment

Rat age (weeks)		Phosphodiesterase $(pmol \cdot min^{-1} \cdot mg^{-1})$	
		Control	+ Trypsin $(10 \mu g/ml)$
4 (n=3)	Basal	55 ± 2	218 ± 17
	+ Insulin	163 ± 16[a]	202 ± 21
8 (n=3)	Basal	45 ± 1	173 ± 39
	+ Insulin	138 ± 6[a]	226 ± 33
16 (n=3)	Basal	48 ± 2	234 ± 19
	+ Insulin	101 ± 8[a]	222 ± 18

Results expressed as mean ± SEM. [a] $p < 0.001$ basal enzyme activity versus enzyme activity + 3 nmol/l insulin in each group

The effects of increased insulin concentrations on phosphodiesterase activity were examined in the three groups. When expressed per mg protein, the enzyme activities in fraction P-2 (Fig. 1 A) of rats aged 4 and 8 weeks were significantly higher at 0.1–30 nmol/l insulin concentrations than that of rats aged 16 weeks ($p < 0.001$). Dose-response curves in the three groups were biphasic and of the convex type, as reported previously [6, 10, 11]. When the data were plotted as a percentage of maximal insulin effect (Fig. 1 B), the dose-response curve in rats aged 16 weeks shifted to the right, and half-maximum stimulations were seen at 0.08 ± 0.01 nmol/l in rats aged 4 weeks, 0.15 ± 0.02 nmol/l in rats aged 8 weeks and 0.22 ± 0.03 nmol/l in rats aged 16 weeks ($p < 0.05$ to $p < 0.01$). When the data were analyzed as a percentage of basal enzyme activity, the maximal insulin effect was approximately 320% at 1 nmol/l in rats aged 4 weeks, 260% at 3 nmol/l in rats aged 8 weeks and 140% at 3 nmol/l in rats aged 16 weeks, respectively (Fig. 1 C). A lower maximal insulin effect was noted in fat cells from rats aged 16 weeks ($p < 0.001$). When the enzyme activities were expressed per cell number (Fig. 1 D), a similar dose-response curve was obtained. However the basal enzyme activity in rats aged 4 weeks was higher than that of rats aged 8 or 16 weeks ($p < 0.001$). These data indicate a decrease in
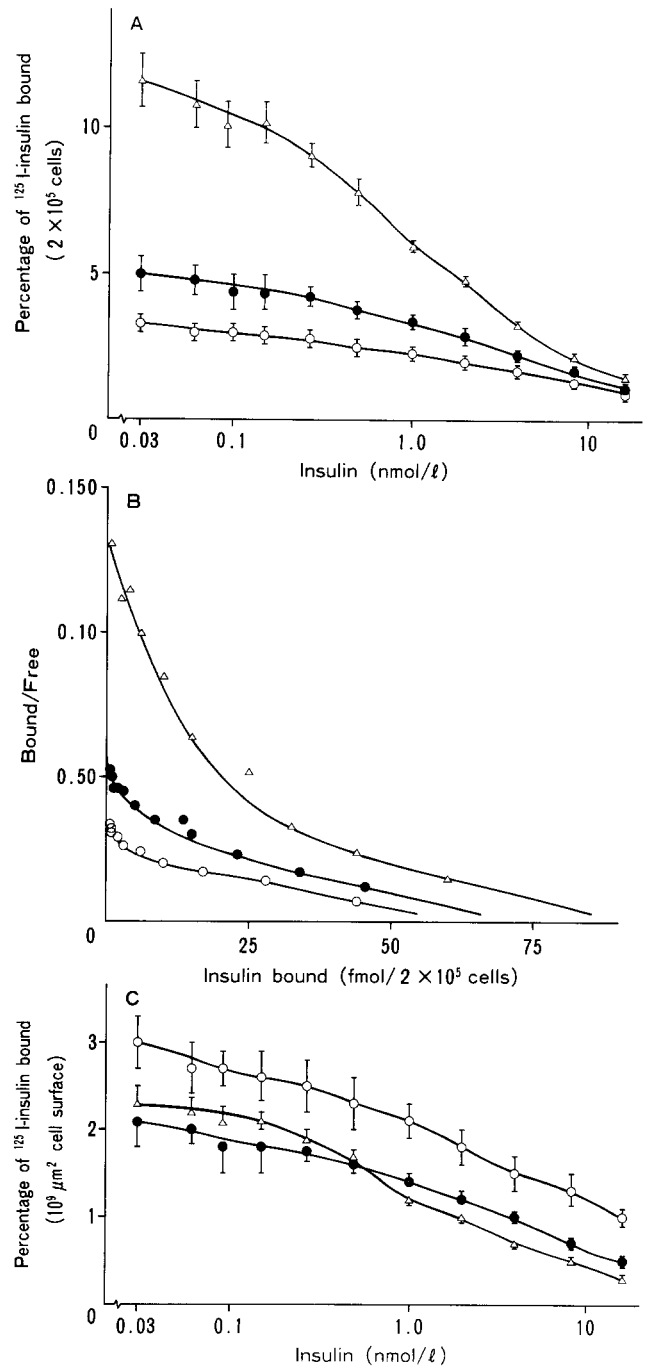
Fig. 3. A-C Binding of radioactive insulin to fat cells from rats aged 4 (○), 8 (●) and 16 (△) weeks. Each symbol and bar show mean ± SEM (n=3). **A** Specific binding at increasing insulin concentration. **B** Scatchard plot of data from (A), **C** Specific binding per fat cell surface area

both sensitivity and responsiveness to insulin in fat cells from obese rats.

Kinetic analysis of phosphodiesterase activity in fat cells from the three groups are presented in Figure 2. Km values for the enzymes in control and insulin-treated fat cells from the three groups were 0.19–0.22 μmol/l, respectively. The values were similar for the enzymes in the three groups. Maximum velocity (V_{max}) values in control and insulin-treated fat cells were 167 ± 9
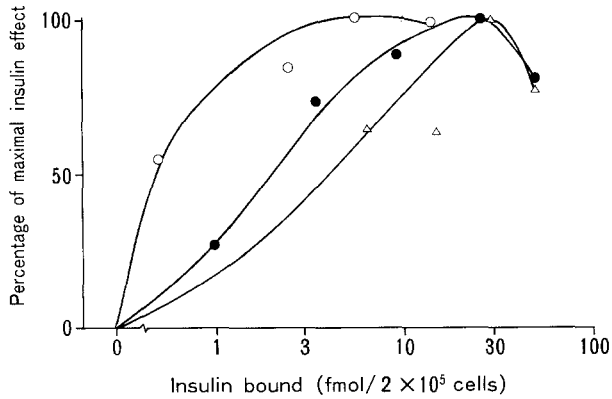
Fig. 4. The relationship between the amount of insulin bound and insulin action. The amount of insulin bound in Figure 3 A is plotted as a function of percentage of maximal insulin effect at that insulin concentration for fat cells from rats aged 4 (O), 8 (●) and 16 (△) weeks

and 446 ± 17 pmol·min^{-1}·mg^{-1} for rats aged 4 weeks, 169 ± 18 and 405 ± 43 pmol·min^{-1}·mg^{-1} for rats aged 8 weeks, 114 ± 7 and 271 ± 4 pmol·min^{-1}·mg^{-1} for rats aged 16 weeks, respectively. The V_{max} values in control and insulin-treated fat cells for rats aged 16 weeks were significantly lower than those for rats aged 4 and 8 weeks ($p < 0.001$). These results indicate that insulin does not affect the K_m, but only the V_{max} values. The enzyme activities of basal plus insulin after mild trypsin treatment were increased to approximately the same levels in the three groups (Table 2).

Specific binding of ^{125}I-insulin to fat cells prepared from rats aged 4, 8 and 16 weeks is shown in Fig. 3 A. The binding of insulin in fat cells from rats aged 16 weeks was significantly higher at all insulin concentrations ($p < 0.001$). At the lowest insulin concentration (0.03 nmol/l), specific binding of insulin to fat cells of rats aged 4, 8 and 16 weeks were $3.3 \pm 0.3\%$, $5.0 \pm 0.6\%$ and $11.6 \pm 0.9\%$ per 2×10^5 cells, respectively. Scatchard analysis is depicted in Fig. 3 B. The curves are curvilinear for fat cells from the three groups. Although it is difficult to calculate the exact binding affinity from curvilinear Scatchard plots, the slopes of the curves indicate that the overall binding affinity greatly increased at 16 weeks of age. Since the horizontal intercepts of these plots represent the total number of receptor sites, the data show that the receptor numbers per cell slightly increased as the cell size increased. Thus, an increase in specific binding of insulin in fat cells from rats aged 16 weeks may be due mainly to an increase of binding affinity. If the data are analyzed per cell surface area (Fig. 3 C), specific binding of insulin to fat cells from rats aged 16 weeks was no more than those of the other groups.

The amount of insulin bound per cell is plotted as a function of the percentage of maximal insulin effect in Figure 4. For a given amount of insulin bound, activation of phosphodiesterase in fat cells from rats aged 16 weeks is less than those in fat cells from rats aged 4 and 8 weeks.

Discussion

We found that in spontaneously obese rats activation of insulin-sensitive phosphodiesterase in fat cells is impaired, despite an increase in the specific binding of insulin. According to Kahn [3], this impairment is due to a decrease in both insulin sensitivity and responsiveness. As we reported previously [9, 10] and is generally accepted [19], the insulin receptor on the plasma membrane is required for activation of phosphodiesterase by insulin. This enzyme seems to be located in the endoplasmic reticulum [6]. We found parallel increases or decreases in insulin binding and sensitivity in streptozotocin-diabetic [11] and adrenalectomized- and dexamethazone-treated rat fat cells [12]. However, as shown in Figure 4, post-receptor defects, namely impaired coupling of the phosphodiesterase system are suggested. Since we found a similar phenomenon in fat cells of spontaneously diabetic KK mice (unpublished observations), it is feasible that the uncoupling of the phosphodiesterase activation system may be a common feature of insulin resistance in obesity.

Although the mechanism is unknown, limited proteolysis by trypsin markedly activated phosphodiesterase as described elsewhere [14]. Thus, enzyme activation with mild trypsin treatment (Table 2) probably reflects the total amount of the enzyme. Since the results from the three groups of rats were similar, it is feasible that the total amount of the enzyme may be similar in the three groups. Kinetic analysis (Fig. 2) showed that the properties of the enzyme in fat cells from the three groups were similar. From these results, decreased activation of the enzyme by insulin in fat cells from obese rats may be due to coupling defects but not to changes of the enzyme itself.

Lockwood et al. [20] reported that stimulation of glucose oxidation by insulin was reduced in large fat cells from obese rats, but when rapid and direct measurements of sugar uptake were made, insulin stimulation was virtually identical in small fat cells from lean rats and large fat cells from obese rats. These findings indicate that the apparent hormone resistance may be due to alterations in intracellular glucose metabolism. However, Hissin et al. [21] reported decreases in intracellular glucose transport systems in large fat cells, and this could lead to impaired glucose transport and insulin resistance. In the present study, the enzyme activity expressed on a per cell basis was decreased in fat cells from rats aged both 8 and 16 weeks, compared with rats aged 4 weeks (Fig. 1 D). However, responsiveness expressed as a percentage of the basal rate was significantly decreased only in the fat cells from rats aged 16 weeks (Fig. 1 C). Thus, the impairment of responsiveness in large fat cells from obese rats cannot be explained simply by a decrease of enzyme activity. Further studies are needed.

All animal models of obesity are characterized by hyperinsulinaemia and normal or increased blood glu-

cose. Since increased insulin levels have been demonstrated to down-regulate the insulin receptor [22], it would be expected that tissues from hyperinsulinaemic animals would have a decreased number of insulin receptors. Insulin receptor binding has been studied extensively in obesity, and significant decreases in the insulin receptor have been demonstrated [23, 24]. It has been shown also that insulin binding is normal [25] or increased in large fat cells [26, 27], and it is clear that defects in intracellular metabolism of large fat cells are most important in relation to insulin resistance. Our data are compatible with the latter. It is unclear why these discrepancies of insulin receptor binding occur, but it may be due to different experimental conditions or animals. In clinical obesity, some authors have shown reduced insulin binding per fat cell [28], while others could not support this hypothesis [29].

Our findings suggest that insulin effector systems linking receptor binding and phosphodiesterase activation are altered in fat cells from spontaneously obese rats and that both sensitivity and responsiveness to insulin decrease. Thus, in these fat cells impairment of the phosphodiesterase activation system is predominantly due to post-receptor defects.

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