Attenuated adenosine-sensitivity and decreased adenosine-receptor number in adipocyte plasma membranes in human obesity

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Fat-cells were isolated from patients of body-mass indices (BMIs) ranging from 17.9 to 83.9 kg/m². Isoprenaline-stimulated cyclic AMP accumulation in cells prepared from obese subjects as compared with normal-weight subjects, was less sensitive to inhibition by the adenosine agonist N⁶-(phenylisopropyl)adenosine (PIA) ($P = 0.047$). The inhibition of 7β-desacetyl-7β-[γ-(N-methylpiperazino)butyryl]-forskolin-stimulated adenylate cyclase by PIA in the presence of adenosine deaminase was also much attenuated in crude plasma membranes of adipocytes prepared from massively obese patients as compared with lean controls ($P = 0.0143$). This difference was probably not due to different cell size, because adenylate cyclase of crude plasma membranes of large adipocytes was actually more sensitive to PIA than was adenylate cyclase of membranes of smaller fat-cells isolated from the same individual. The stimulatory effect of PIA on glucose uptake in the presence of adenosine deaminase was depressed in adipocytes prepared from obese subjects and correlated with BMI at $r = -0.626$ ($P = 0.007$) at 100 nM-PIA. The adenosine receptors were studied by using the adenosine antagonist 1,3-[³⁵H]dipropyl-8-cyclopentylxanthine. The binding was rapid and proportional to protein concentration. There was no difference in the affinities of receptors in membranes of obese and normal-weight subjects; $K_a$ values of all patients averaged 3.3 nm. $B_{max}$ values were 54 and 130 fmol/mg of protein in membranes prepared from seven obese and five control patients respectively. The $B_{max}$ values calculated per mg of protein correlated with BMI at $r = -0.539$ ($P = 0.047$). The adenosine content of adipose tissue was higher in obese than in control subjects. These results demonstrate an attenuated response of cyclic AMP accumulation, adenylate cyclase and glucose uptake to adenosine in fat-cells prepared from obese subjects, and suggest that this change is at least partly due to changes in the amount of adenosine receptors, but not their affinity. The decreased receptor number could be due to higher adenosine content. A higher adenosine concentration in adipose tissue could explain why lipolysis is inhibited in situ in obesity, and the desensitization could explain the diminished response to adenosine analogues in isolated fat-cells.

INTRODUCTION

Morbid obesity is associated with profound metabolic changes. Studies on the turnover of labelled non-esterified fatty acids in plasma show that lipolysis, the release of non-esterified fatty acids and glycerol from adipocytes, is inhibited in vivo in obese subjects (Lillioja et al., 1986). However, fat-cells isolated from obese donors and incubated in vitro have shown to have an increased basal lipolysis and to display an attenuated response to inhibitors of cyclic AMP accumulation and lipolysis such as adenosine (Ohisalo et al., 1986), clonidine and prostaglandins (Richelsen, 1988). The molecular basis of this phenomenon has not been elucidated.

Lipolysis is accomplished by the enzyme hormone-sensitive lipase, which is activated by phosphorylation of protein kinase A, which, in turn, is activated by cyclic AMP. Thus lipolysis is controlled by receptors which inhibit or activate adenylate cyclase via the inhibitory and activating guanine-nucleotide-binding proteins (G-proteins) $G_i$ and $G_s$ (Gilman, 1987). β-Adrenergic receptors activate, whereas α-adrenergic and adenosine A1 receptors inhibit, the cyclase. Adenosine is a short-lived nucleoside that exerts a local effect on adipocyte metabolism, inhibiting adenylate cyclase and thereby release of glycerol and non-esterified fatty acid (Schwabe et al., 1973; Ohisalo et al., 1984). Adenosine also stimulates lipoprotein lipase activity (Ohisalo et al., 1981; Kern et al., 1988) and glucose uptake (Schwabe et al., 1973; Stoneham, 1989). It was first reported by Ohisalo & Stouffer (1979) that the sensitivity of adipocytes to adenosine is modulated by thyroid hormones, cells from hypothyroid rats being more sensitive to inhibition of lipolysis by the adenosine-receptor agonist N⁶-(phenylisopropyl)adenosine (PIA). This altered sensitivity has been confirmed by several groups (Malbon et al., 1985; Woodward & Saggerson, 1986; Rapiejko & Malbon, 1987). Changes in the sensitivity to adenosine have been reported since then in hypocortisolism (Saggerson, 1980), with aging (Hoffman et al., 1984), and both in human obesity (Ohisalo et al., 1986) and later in genetically obese rodents (Vannucci et al., 1989; Bégin-Heick, 1990). Most investigators have suggested that alterations in G-protein levels or activity explain such changes. Clearly, the switch to more active lipolysis and weakened inhibitory responses could be due to any step from the affinities and amounts of receptors to the lipase itself, or to the phosphodiesterases that degrade cyclic AMP.

The aim of this work was to characterize the changes in the regulation of adipose-tissue metabolism in human obesity and to find the molecular basis of such changes.

MATERIALS AND METHODS

Patients

Samples were taken from human subjects of body-mass index (BMI) ranging from 17.9 to 83.9 kg/m². The BMI is obtained by dividing weight (kg) by the square of height (m), and values of 20–25 kg/m² are considered normal. The subjects with the highest BMIs were patients who underwent a gastric bypass operation, during which the samples were taken. This operation

Abbreviations used: PIA, N⁶-(phenylisopropyl)adenosine; BMI, body-mass index; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine.

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restricts the maximum energy intake by mechanical means and induces a dramatic weight loss. The BMIs of these patients averaged 55 kg/m², and adequate samples could easily be excised. It is more difficult to obtain enough fat from normal-weight subjects. However, large fat samples were obtained from patients who had been obese but had become normal weight or almost normal weight (average BMI 28 kg/m²) after a gastric bypass and had reached a stable body weight. Such a considerable loss of weight leaves the abdominal skin loose, requiring a reconstructive plastic operation. Also, many such patients develop gallstones that need to be removed by cholecystectomy. The samples from the post-bypass patients were taken at such operations. The other normal-weight patients had abdominal operations and donated adipose-tissue samples at that time. All the samples were taken from subcutaneous adipose tissue by open surgical biopsy under general anaesthesia in the morning after an overnight fast. Glucose was not infused. A wedge-shaped sample of subcutaneous abdominal adipose tissue was excised at the beginning of the operation and placed in warm iso-osmotic saline solution for transportation to the laboratory.

Preparation of fat-cells and crude plasma membranes

Fat-cells were isolated by a modification of the method of Rodbell (1964) in the presence of collagenase (2 mg/ml) under constant shaking at 2 Hz at 37 °C in a buffer containing 125 mM-NaCl, 5 mM-KCl, 1 mM-CaCl₂, 2.5 mM-MgCl₂, 1 mM-KH₂PO₄, 4 mM-glucose, 2 %, BSA, and 25 mM-Tris at pH 7.4. After 20 min, the cells were filtered through a nylon cloth and washed three times with the same buffer without collagenase. To obtain crude plasma membranes, the cells were homogenized in 2 vol. of 150 mM-NaCl/1 mM-EDTA/10 mM-Tris, pH 7.4, in the presence of leupeptin and phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 100000 g for 1 h, and the pellet was suspended in the same buffer (with phenylmethylsulfonyl fluoride and leupeptin) at about 1 mg of protein/ml. For glucose-uptake studies, cells were isolated as described by Stoneham (1989), with 2 mM-glucose in the collagenase treatment and glucose-free buffer in the washes.

Attempts were made to separate cells of different sizes from the same individual by sieving with nylon cloths of different mesh sizes, but they were unsuccessful. Small preparations of cells of different sizes could, however, be made by first separating the largest cells that rose to the top of the fat-cell layer in long thin plastic tubes. Intermediate-size cells were discarded and the small cells were taken from the lowest part of the fat layer that floated immediately.

Studies of cyclic AMP accumulation

For this, 100 μl of isolated fat-cells was incubated for 60 min in 1 ml of the same buffer at 37 °C with constant shaking at 1 Hz, with the addition of adenine deaminase, 1 μM-isoprenaline (isoproterenol) and different concentrations of PIA. The incubations were terminated by adding 100 μl of 35 % (w/v) HClO₄, followed by centrifugation and neutralization or by boiling the samples for 2 min. Cyclic AMP was assayed by radioimmunnoassay with a kit from Advanced Magnetics Inc. (Cambridge, MA, U.S.A.).

Adenylate cyclase assay

Adenylate cyclase activity of fresh adipocyte crude membranes was assayed in a total volume of 300 μl in the presence of 150 mM-NaCl, 10 mM-KCl, 10 mM-MgCl₂, 1 mM-EDTA, 2 mM-dithiothreitol, 1 mM-ATP, 100 μM-papaverine, 1 mM-GTP, BSA (1 mg/ml), 50 mM-Tris/HCl, pH 7.5, and adenine deaminase (1 μg/ml). 7β-Desacetyl-7β-[y-(N-methylpiperazino)butyryl]-forskolin (10 μM) and different concentrations of PIA were added as shown. After 20 min at 37 °C, the incubation was terminated by boiling the tubes for 2 min. The boiled samples were then centrifuged and assayed for cyclic AMP as described above.

Glucose uptake

This was studied by the method of Kashiwagi et al. (1983) in cells isolated in the presence of 2 mM-glucose and then washed in glucose-free medium. The tracer was 580 nM-[³¹⁴C]glucose. The incubations were done in a total volume of 400 μl in the presence of exogenous adenosine deaminase for 60 min under constant shaking at 37 °C and were started by addition of the cells in 250 μl. At 0 and 60 min, 150 μl samples were taken and pipetted to plastic microcentrifuge tubes containing dimethyl phthalate, whose density is between that of fat-cells and water. The tubes were centrifuged in a Beckman Spinco 152 microcentrifuge as described by Gliemann & Sonne (1978). The tubes were cut through the oil layer, and the upper parts containing the cells were counted for radioactivity.

Receptor-binding experiments

Adenosine A₁-receptors were characterized by using the adenosine antagonist 1,3-[³¹¹H]dipropyl-8-cyclopentylxanthine ([³¹¹H]DPCPX) as the radioactive ligand and 10 μM unlabelled PIA to measure non-specific binding. The assay was performed at 30 °C in a total volume of 250 μl of 100 mM-NaCl/10 mM-MgCl₂/1 mM-EDTA/10 mM-Tris, pH 7.4. The assay mixture also contained 40 μM 5'-guanylylimidodiphosphate. After 45 min of incubation, the mixtures were poured over glass-fibre filters under suction by a vacuum of 15 mmHg. The filters were washed with 2 x 5 ml of water, and the radioactivity associated with the filters was counted. Kᵣ values for each individual patient were first determined by computer, and in computing the Bₘₐₓ. the geometric mean of all Kᵣ values obtained was used.

Assay of adenosine content of adipose tissue

Small adipose-tissue pieces were excised from each patient at the beginning of the operation. The tissue pieces were frozen immediately in liquid nitrogen and processed as described elsewhere (Ranta et al., 1985; Stoneham et al., 1988). After freeze-drying of the samples, adenosine was measured by radioimmunoassay using an antisera raised in a rabbit against laevulinic acid-(O²⁻³⁻-adenosine-acetamidalbumin conjugate (Ranta et al., 1985).

Statistical analyses

These were performed by computer using the Macintosh SA Statview 512 program.

Reagents

[³¹¹H]DPCPX (102 Ci/mmol) was from Amersham, and [U-³¹⁴C]glucose (341 Ci/mmol) and [²,₈,₅⁻³¹⁴C]adenosine (50.5 Ci/mmol) were from New England Nuclear, Boston, MA, U.S.A. Adenosine deaminase (type VIII from calf intestine), PIA, fatty-acid-free BSA (fraction V) and isoprenaline hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and collagenase was from Worthington Biochemicals. The adenosine antisera was kindly provided by Dr. Andrew C. Newby, Cardiff, Wales, U.K. 7β-Desacetyl-7β-[y-(N-methylpiperazino)butyryl]-forskolin was from Calbiochem, San Diego, CA, U.S.A. and the cyclic AMP assay kit (cat. no. 6301) from Advanced Magnetics, Cambridge, MA, U.S.A.

RESULTS

Fat-cells release adenosine into their incubation medium, and for studies of adenosine responsiveness this endogenous adenosine has to be removed. In addition, cells take up added
Adenosine-sensitivity of adipocytes in obesity

Fig. 1. Cyclic AMP levels of human adipocytes at different times after stimulation by forskolin or isoprenaline

Fat-cells were isolated and incubated in the presence of adenosine deaminase and either 10 μM-7β-desacetyl-7β-[γ-(N-methylpiperazino)butyryl]-forskolin or 1 μM-isoprenaline for different periods of time. The level at 40 min in the presence of the forskolin analogue was designated 100%. The results are means of three independent experiments with fat-cells from three different donors. ●, 7β-Desacetyl-7β-[γ-(N-methylpiperazino)butyryl]-forskolin; ○, isoprenaline.

Fig. 2. Inhibition of cyclic AMP accumulation in fat-cells isolated from subjects with BMI above and below 30 kg/m²

Isolated fat-cells (100 μl of cells/ml) were incubated in the presence of adenosine deaminase and isoprenaline for 60 min as described in the text. Different amounts of PIA were added as shown. The level of cyclic AMP in the absence of the adenosine analogue was designated 100%. Vertical bars indicate S.E.M. □, Obese [BMI 64.1 ± 7.2 kg/m² (mean ± S.E.M.; n = 4)]; ■, lean (BMI 23.7 ± 7.2 kg/m²; n = 5).

Adenosine and deaminase and phosphorylate it. These problems can be overcome by adding adenosine deaminase to remove endogenous adenosine and using PIA, which is an adenosine A1-receptor agonist but is neither a substrate nor an inhibitor of adenosine deaminase. For studies of cyclic AMP levels, a time point first had to be chosen. Fig. 1 shows the cyclic AMP levels as a function of time in adipocytes stimulated by forskolin and isoprenaline. The levels rose rapidly and then remained constant, in contrast with reports on rat fat-cells, where the levels reach a peak and then decline. The levels remained constant for at least 80 min. Therefore the studies reported below were done at 60 min of incubation.

The inhibition of isoprenaline-stimulated cyclic AMP accumulation by PIA in adipocytes of four obese (BMI 46.4–81.0 kg/m²; mean 64.1) and six lean or normal-weight subjects (BMI 17.9–29.0 kg/m²; mean 23.7) at 60 min of incubation are shown in Fig. 2. The effect of the nucleoside analogue was clearly more pronounced in the cells prepared from normal-weight donors (P = 0.047 with repeated-measures analysis of variance).

The differences in the levels of cyclic AMP in fat-cells from different subjects could be due to changes in the regulation or amount of adenylate cyclase or cyclic AMP phosphodiesterase. Studies with isolated plasma membranes revealed that adenylate cyclase activity stimulated by 7β-desacetyl-7β-[γ-(N-methylpiperazino)butyryl]-forskolin was more responsive to inhibition by PIA in membranes prepared from lean (BMI 18.8–28.4 kg/m²; n = 4) as compared with membranes from obese (BMI 38.4–83.9 kg/m²; n = 6) patients (Fig. 3). The dose–response curve was shifted to the right about 20-fold in membranes prepared from obese as compared with lean subjects. This difference was statistically significant at P = 0.014 (repeated-measures analysis of variance).

Since it has been suggested that cell size affects the hormone response of fat-cells directly (Arner et al., 1987), we prepared cells of different sizes from the same subject. Crude membranes were isolated from both large (460–730 pl) and small (250–430 pl) fat-cells of three individuals with BMIs ranging from 27.1 to 30.1 kg/m². The large cells were on average 1.8 times larger in volume than the corresponding small cells. Adenylate cyclase activity of the membranes was stimulated by 7β-desacetyl-7β-[γ-(N-methylpiperazino)butyryl]-forskolin in the presence of adenosine deaminase, and different concentrations of PIA were then added. The dose–response curves are summarized in Fig. 4, which shows that membranes prepared from large cells were in fact more responsive to the nucleoside analogue than were those from smaller cells.

Glucose uptake as measured by the tracer method was linear up to 90 min of incubation and was decreased by removal of endogenous adenosine by adding adenosine deaminase. In the presence of this enzyme, glucose uptake could then be stimulated by low concentrations of PIA. The stimulation of glucose uptake by PIA in the presence of adenosine deaminase in cells prepared from lean and obese subjects is shown in Fig. 5. The stimulatory
effect on glucose uptake was less in cells prepared from obese (BMI 32.5–52.8 kg/m²) as compared with normal-weight controls (BMI 20.0–31.2 kg/m²). Since all data points were not available on all the patients, a simple analysis of variance could not be applied, but the difference was statistically significant at \( P = 0.0077 \) at 100 nm-PIA (Student’s two-tailed test of independent values). There was also a correlation between BMI and the effect of the adenosine analogue on glucose uptake at \( r = -0.626 \) and \( p = 0.007 \) (Fig. 6).

Adenosine receptors were studied by using \(^{3}H\)DPCPX. This compound is an adenosine antagonist. The binding of DPCPX to human fat-cell membranes was rapid in the presence of 5'-guanylylimidodiphosphate, and it was directly proportional to the amount of membrane protein used. Specific binding was approx. 50\% at 1 nm-DPCPX. One binding experiment is displayed in Fig. 7. Altogether 14 determinations of \( K_s \) values using samples from different patients were done. The \( K_s \) values were calculated by computer with a program that gives the same weight to each data point. There was no difference between the obese and lean groups. When computing the \( B_{\text{max}} \) values, the geometric mean value of 3.3 nm was used as the \( K_s \) for all samples. The \( B_{\text{max}} \) values of plasma-membrane adenosine receptors as a function of the BMI of the donor are displayed in Fig. 8. Clearly, the receptor density calculated per mg of protein was lower in the obese than in the normal-weight subjects. There was a correlation at \( r = -0.539 \), which was statistically significant at \( P = 0.047 \) (regression analysis). The \( B_{\text{max}} \) values of five normal-weight (BMI 20.3–29.8 kg/m²) and seven obese (BMI 38.6–81.0 kg/m²) subjects were 130 ± 29 and 54 ± 8 fmol/mg of protein, respectively (means ± s.e.m.). The difference was significant at \( P < 0.018 \) (Student’s two-tailed \( t \) test of independent values).
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The adenosine content of abdominal subcutaneous adipose tissue was measured in 35 patients. The adenosine content of the adipose tissue of 11 obese patients (average BMI 50.4 kg/m²) was 0.67 ± 0.14 pmol/g wet wt. (mean ± S.E.M.) as compared with 0.42 ± 0.06 pmol/g wet wt. in 24 control patients (average BMI 24.4 kg/m²), giving P = 0.058 in Student's two-sided t-test of unpaired values.

**DISCUSSION**

These results confirm our earlier finding of an attenuated response to PIA as an inhibitor of isoprenaline-stimulated cyclic AMP accumulation in fat-cells isolated from obese donors as compared with normal cells (Ohisalo et al., 1986). A similarly decreased response to inhibition via the α-adrenergic receptor in obesity was reported by Arner et al. (1987), whereas the stimulatory response mediated via the β-adrenergic receptor has been reported to be unchanged (Arner et al., 1987) or even decreased (Martin et al., 1990). Therefore it seems that the alterations observed in the regulation of adipocyte metabolism in obesity can be explained by an altered inhibition of cyclic AMP accumulation, but not by enhanced stimulatory responses. The attenuated response to PIA in obesity was also observed with crude fat-cell membranes in the presence of an inhibitor of cyclic AMP phosphodiesterase. This suggests that a change in the activity of this enzyme cannot by itself explain the alterations in cells isolated from obese donors. The response in the isolated membranes was studied by using 7β-desacetyl-7β-[N-methyl-lipiperazino]butyryl]-forskolin to stimulate adenylyl cyclase directly independent of receptor systems. Therefore, the sluggish response of adenylyl cyclase to inhibition by PIA cannot be due to high activity of the stimulatory arm of the regulatory system. This concept is further strengthened by the finding that glucose uptake in the presence of adenosine deaminase, but no other pharmacological agents, was less sensitive to stimulation by PIA in fat-cells from obese donors. This response is thought to be independent of cyclic AMP accumulation, though a G-protein seems to be involved (Kuroda et al., 1987). The altered response could be explained by altered receptor number or affinity, an alteration in G-proteins or in the interaction between receptor and G-protein. Finally, it could be due to an alteration in the interaction of G-protein both with adenylyl cyclase and with the second-messenger systems that lead to increased glucose uptake. An alteration occurring in the regulatory cascades before their point of divergence would be the simplest explanation for both changes, and this would place the molecular alteration at the receptor/G-protein level.

The present results show that the affinity of the adenosine receptor of crude adipocyte membranes for DPCPX is not affected by obesity. The receptor number as calculated per mg of protein was clearly decreased in obesity and negatively correlated with the BMI of the donor. Can this, then, be interpreted to mean that the attenuated sensitivity to adenosine is due to a decreased receptor density? It must first be noticed that we have previously reported that the amounts of both G₁ and G₃, as calculated per mg of protein, are lower in obesity (Ohisalo & Milligan, 1989) and that the observed relative difference was roughly the same as that reported here for adenosine receptors. On the other hand, only a fraction of G₁ can be bound to adenosine receptors, and we have to wait until we can measure G-proteins bound to adenosine receptors before we can know the significance of the decrease in G₁. If G₁ is present in excess, then a lowering of specific adenosine-receptor density could determine sensitivity to adenosine, despite changes in G-protein levels.

Cell size has been suggested to be a very important determinant of hormone-responsiveness (Arner et al., 1987). Increased lipolysis in large fat-cells could, of course, be a way of keeping adipocytes from growing too large. Neither the present results with crude membranes prepared from adipocytes of different sizes from the same individual, nor the results on co-isolated small and large rat fat-cells reported by Francendese & DeMartinis (1985), support this suggestion, but instead suggest that other factors are involved. One should notice, however, that fat-cells from massively obese donors are larger than the 'large' fat-cells in Fig. 4, and cell size may become more important with very large cells. Unfortunately, very large fat-cells are fragile and could not be separated from smaller cells without excessive cell breakage.

The specific activity of adenylyl cyclase was also lower in crude adipocyte membranes of obese individuals (see legend to Fig. 3). This was not due to different cell size alone, because the forskolin-stimulated maximal adenylyl cyclase activity of membranes prepared from smaller cells was only 77 ± 5% (mean ± S.E.M.) of that of membranes of co-isolated larger cells.

The adenosine content of adipose tissue was higher in obese than in normal-weight individuals, though the large variation at the values and problems in the interpretation of the data (Ranta et al., 1985) make it difficult to evaluate the significance of this finding. An elevated extracellular concentration of adenosine in obesity could explain the inhibition of lipolysis in situ and, through a desensitization mechanism, the decreased sensitivity of adipocytes from obese subjects to this nucleoside in vitro.

In summary, the present results show that both the inhibitory response of human adipocyte adenylyl cyclase and the stimulatory response of glucose uptake to an adenosine analogue are attenuated in obesity, and suggest that this is not due to a change in cell size. The affinity of adenosine receptors was unaltered, but the number of receptors per mg of protein was low in membranes prepared from obese donors. These changes and the discrepancy between inhibited lipolysis in vivo and stimulated lipolysis in vitro could be explained by the observed increased adipose-tissue adenosine content.

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