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Antilipolytic effects of N^6-phenylisopropyladenosine and prostaglandin E_2 in fat-cells of obese volunteers before and during energy restriction

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1. The antilipolytic effects of N^6-phenylisopropyladenosine and of prostaglandin E_2 were studied with adipocytes of obese volunteers before and after 4 weeks of severe energy restriction [1250 kJ (300 cal)/day] in the presence and absence of adenosine deaminase (1.6 μg/ml, corresponding to 320 m-units/ml). 2. The studies were undertaken to define more clearly the role that local modulators might play in adaptation of lipid mobilization to starvation in humans. 3. Starvation was associated with an approx. 3-fold increase in non-stimulated lipolysis. 4. Removal of endogenous adenosine resulted in a similar increase in basal glycerol release under both conditions, averaging 2 and 2.2 μmol/180 min per 10^6 cells respectively. 5. The sensitivity of the cells to N^6-phenylisopropyladenosine and to prostaglandin E_2 was not changed by starvation in the presence of adenosine deaminase. 6. These results are discussed in terms of the possible role that local regulators might play during dietary adaption in human fat-cells *in vitro*.

INTRODUCTION

Fat-cells of various species, including human adipocytes, produce and extrude potent antilipolytic compounds such as adenosine or prostaglandins (Bergström, 1967; Schwabe *et al.*, 1974; Chang *et al.*, 1977; Ohisalo, 1981). During the past few years considerable information has been obtained about the molecular mechanisms of action of these compounds. Fat-cells possess a subclass of adenosine receptors termed A_1- (Van Calker *et al.*, 1979) or R_i-receptors (Londos *et al.*, 1980), which are coupled to adenylate cyclase in an inhibitory fashion (Londos *et al.*, 1980). Prostaglandins inhibit lipolysis via similar mechanisms (Kather & Simon, 1979).

Less is known about the physiological role that local modulators might play in normal fat-cell physiology. However, there is mounting evidence from studies *in vitro* suggesting that endogenous inhibitors are important in long-term regulation of lipid mobilization. In the rat the response of fat-cell lipolysis to adenosine or to its analogue N^6-phenylisopropyladenosine, which is selective for A_1-adenosine receptors, is increased with increasing age (Hoffman *et al.*, 1984), during lactation (Vernon *et al.*, 1983) and in hypothyroid or adrenalectomized animals respectively (Ohisalo & Stouffer, 1979; Saggerson, 1980). In addition, starvation was associated with decreasing sensitivity of fat-cells towards N^6-phenylisopropyladenosine in this species (Chohan *et al.*, 1984).

In human adipose tissue severe energy restriction or total starvation for 1 week or more have been found to be associated with increased rates of basal lipolysis (Kjellberg & Östman, 1971; Lisch *et al.*, 1973; Berlan *et al.*, 1981; Rozen *et al.*, 1984). Concomitantly the lipolytic effects of adrenaline and noradrenaline are impaired or even reversed (Kjellberg & Östman, 1971; Berlan *et al.*, 1981). We have previously shown that these adaptive changes can be mimicked by removal of endogenous adenosine (Kather *et al.*, 1985*a*). We have interpreted these latter findings as suggesting that the increase in basal lipolytic rate *in vitro* seen during starvation is mainly due to relief of endogenous inhibitory influences which could be caused by a decrease in the steady-state concentrations of endogenous inhibitors or by lowered sensitivity and/or responsiveness of human fat-cells to antilipolytic modulators such as adenosine or prostaglandins.

To gain further insight into the role that local modulators might play during dietary adaptation, we have compared the concentrations–response relationships of prostaglandin E_2 and of the A_1-site-selective adenosine analogue N^6-phenylisopropyladenosine in fat-cells from obese volunteers before and after 28 days of severe energy restriction.

PATIENTS AND METHODS

Subjects

Some 17 obese subjects of both sexes were investigated (nine females and eight males). They were otherwise healthy and their age was 37 ± 10 years. Fasting blood glucose was normal. None was taking drugs known to interact with adipose-tissue metabolism, and none showed evidence of a recent weight change. All were examined as in-patients and fed on an isoenergetic diet for at least 3 days before the first biopsy. Thereafter they received an energy-restricted diet (1250 kJ (300 cal)/day] for 28 days. During starvation they lost 10 ± 2 kg in weight. In order to avoid fluid retention, four of the subjects received 50 mg of spironolactone during the last 2 weeks of starvation. Three of the subjects were also treated with allopurinol. The nature, purpose and possible risks of the study were explained to all subjects involved before their consent to participate was obtained. The study was approved by the local Ethical Committee.

Biopsies, cell preparation and incubations

Adipose tissue was removed between 09:00 and 10:00 h after an overnight fast for 12–14 h. Tissue specimens were

obtained from periumbilical region (7–10 cm lateral from the umbilicus) by needle biopsies, which were carried out, after intracutaneous anaesthesia with lidocaine, with needles of 2 mm diameter and a 20 ml syringe containing 2 ml of 0.9% NaCl. After aspiration of approx. 300 mg of tissue, fat-cells were isolated by the method of Rodbell (1964) in Krebs–Henseleit (1932) bicarbonate buffer, pH 7.4, containing 40 g of human serum albumin/l, 5 mM-glucose and 2 mg of collagenase (Sigma, type II)/ml. After incubation for 45 min at 37 °C, cells were freed from connective tissue by filtering through a nylon screen and washed three times by floating. The cells were resuspended at a final concentration of 10000–20000 cells/ml in the same medium, except that collagenase was omitted.

Samples of the cell suspension (50 μl; 500–1000 cells) were incubated in stoppered plastic vials (2 ml) under O_2/CO_2 (19:1) for 180 min. Incubations were stopped by heating. Fat-cell number was determined by counting all cells in appropriately diluted samples (10 μl) of the cell suspension.

Determination of glycerol

Glycerol release was determined by a bioluminescence method using an automatic luminescence analyser (Berthold LB 950 T). A manual version of this method has been described in detail elsewhere (Kather *et al.*, 1982; Kather & Wieland, 1984). The use of an automatic analyser required some minor modifications. Briefly: 0.02 ml of the appropriately diluted sample (2–20-fold) was added to an equal volume of a medium composed of 28 mM-triethanolamine/HCl, pH 9.0, 1.1 mM-KH_2PO_4, 20 mM-Na_3AsO_4, 1.1 mM-dithiothreitol, 2.9 mM-$MgCl_2$, 1.54 mM-ATP, 8.0 mM-NAD^+, 7 units of glycerokinase/ml, 2.3 units of glycerol-3-phosphate dehydrogenase/ml, 19 units of glyceraldehyde-3-phosphate dehydrogenase/ml and 33 units of triose-phosphate isomerase/ml. After incubation for 120 min at 37 °C, the samples were further diluted (6-fold) and 0.01 ml portions of the diluted media were assayed for NADH.

The assay cocktail of the luciferase reaction contained 0.5 mM-tetradecanal, 1.1 μM-FMN, 15 units of bacterial luciferase/l and 85 units of NAD(P)H:FMN oxidoreductase/l. The assay cocktail was prepared daily and kept at +2 °C during measurements.

Light production was measured by a microcomputer-controlled automatic luminescence analyser. The vials, containing 0.01 ml of sample, were automatically moved into the measuring position. The reaction was started by automatic injection of 0.25 ml of the assay cocktail. Measuring time was 15 s. The integral of counts between 10 and 15 s was taken as a measure of NADH concentration.

Chemicals

Collagenase (type II) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Prostaglandin E_2 was from Serva A.G., Heidelberg, W. Germany. Highly purified human serum albumin was from Behring-werke, Marburg, W. Germany. Enzymes, coenzymes and N^6-phenylisopropyladenosine were from Boehringer Mannheim, Mannheim, W. Germany. $(NH_4)_2SO_4$ was removed from enzyme suspension by centrifugation.

RESULTS AND DISCUSSION

The results of the present studies confirm previous findings that human fat-cell lipolysis is increased during starvation (Kjellberg & Östman, 1971; Lisch *et al.*, 1973; Berlan *et al.*, 1981; Rozen *et al.*, 1984; Östman *et al.*, 1984; Kather *et al.*, 1985*a*). Before starvation, basal glycerol release was low, averaging 1–1.5 μmol/180 min per 10^6 cells (Fig. 1*a*, Table 1). A maximally effective concentration of isoprenaline (1 μM) caused a more than 5-fold increase in glycerol release under these conditions (Fig. 1*a*). Severe energy restriction was associated with a

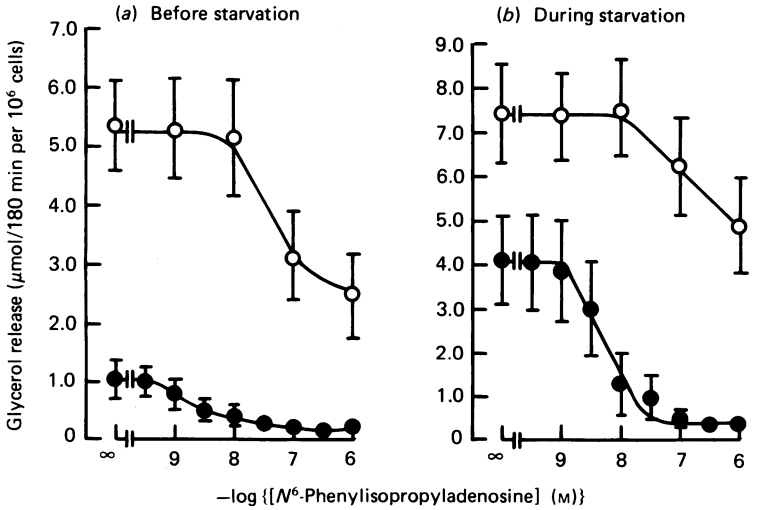

Fig. 1. Effects of N^6-phenylisopropyladenosine on basal and isoprenaline-stimulated glycerol release before and during energy restriction

Values are means ± S.E.M. for four (isoprenaline) or seven (basal glycerol release) paired experiments carried out in duplicate with fat-cells of different subjects under both conditions. Points lacking bars indicate that the S.E.M. did not exceed the size of the symbols. ●, Basal lipolysis; ○, isoprenaline (1 μM).

Table 1. Effects of adenosine deaminase on non-stimulated lipolysis before and during energy restriction

Values are means ± S.E.M. of duplicate determinations carried out in 12–15 subjects under both conditions as indicated in the column '*n*'. *Significant differences between the fed and the starved ($P \leq 0.05$, Wilcoxon test). †Significant difference between controls and samples containing adenosine deaminase ($P \leq 0.05$, Wilcoxon test).

Addition	Glycerol release (μmol/180 min per 10^6 cells)		*n*
	Before starvation	During starvation	
None	1.3±0.3	3.5±0.5*	15
Adenosine deaminase (1.6 μg/ml)	3.3±0.5†	5.7±0.6†	12

marked increase in glycerol release, averaging 3.8–4.0 μmol/180 min per 10^6 cells (Fig. 1*b*, Table 1), which is probably important physiologically because serum concentrations of non-esterified fatty acids and of glycerol are also elevated under these conditions (Hales *et al.*, 1978). In the presence of isoprenaline (1 μM), however, the rate of lipolysis was similar for fat-cells from fed and starved donors. Because mainly basal lipolysis was increased during starvation, isoprenaline caused a less than 2-fold increase in glycerol release in this condition (Fig. 1*b*).

N^6-Phenylisopropyladenosine inhibited basal glycerol release before and during starvation. The inhibitory effect showed dose-dependency. On average, fat-cells from fed donors appeared to be more sensitive to N^6-phenylisopropyladenosine than were those from starved subjects in the absence of adenosine deaminase. It is difficult, however, to assess the relevance of this observation, because basal lipolytic rates of fat-cells from fed donors were low under these conditions. Accordingly the dose–response curves were flat, making it impossible in four out of seven experiments to determine the concentration of the inhibitor causing half-maximal effects with sufficient accuracy (Fig. 1*a*). During starvation, basal lipolytic rate was increased and reliable dose–response curves were obtained in all subjects studied. Half-maximal effects were observed at slightly less than 10 nM-N^6-phenylisopropyladenosine in these latter experiments (Fig. 1*b*). The same qualitative pattern of effects was observed for prostaglandin E_2, with half-maximal inhibition occurring at approx. 50 pM during starvation (results not shown). These findings are consistent with previous reports showing that N^6-phenylisopropyladenosine and prostaglandin E_2 are potent inhibitors of human fat-cell lipolysis *in vitro* (Ohisalo, 1981; Efendic, 1970).

Isoprenaline-activated rates of glycerol release were more resistant to the inhibitory action of N^6-phenylisopropyladenosine (Fig. 1). Even at the highest concentration employed (1 μM), the inhibitor only partially suppressed glycerol release. N^6-Phenylisopropyladenosine appeared to be more potent in inhibiting isoprenaline-stimulated glycerol release before than during starvation. This difference was not significant statistically.

In the rat the inhibitory effect of N^6-phenylisopropyladenosine is dependent on the concentration of activating catecholamines (Schwabe *et al.*, 1974; Stock & Prilop, 1974; Trost & Stock, 1977). This is also true for human adipocytes (Fig. 2). The medium contained 1.6 μg of adenosine deaminase/ml in this experiments. Fat-cells were obtained after 28 days of starvation. Even

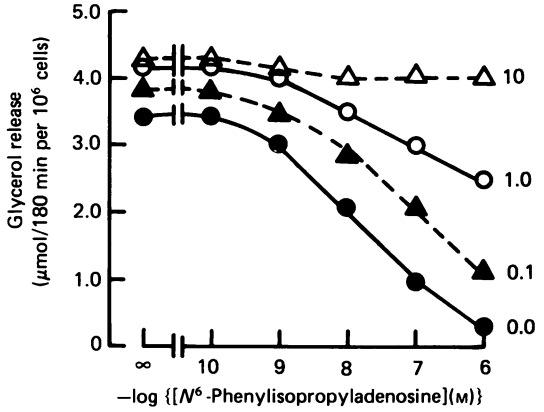

Fig. 2. Effects of increasing concentrations of isoprenaline on the antilipolytic action of N^6-phenylisopropyladenosine

Fat cells were obtained after 28 days of severe energy restriction. The medium contained 1.6 μg of adenosine deaminase/ml. Numbers to the right of the respective dose–response curves denote the concentrations (μM) of isoprenaline. Values are means of duplicate determinations.

maximally effective concentrations of isoprenaline caused only a small increase of lipolytic rate under these conditions. However, the inhibitory action of N^6-phenylisopropyladenosine was progressively decreased with increasing concentrations of isoprenaline and was virtually absent at 10 μM-isoprenaline. The complex mutual interrelationship between the effects of isoprenaline and of N^6-phenylisopropyladenosine tends to complicate comparisons between individuals and studies in the same subjects under different conditions. It is important to choose submaximal concentrations of the catecholamine that are at equivalent points of the isoprenaline dose–response curve in all individuals and under all conditions studied. Human subjects are more heterogeneous genetically than inbred animal strains; therefore sensitivity to isoprenaline is probably more variable in human beings than in laboratory animals. Because of this latter ambiguity, further studies were carried out with adenosine deaminase only, i.e. in the absence of activating catecholamines.

Table 1 shows the effects of adenosine deaminase (1.6 μg/ml) on basal lipolysis before and during starvation. Adenosine deaminase increased basal glycerol release of adipocytes obtained before starvation to values

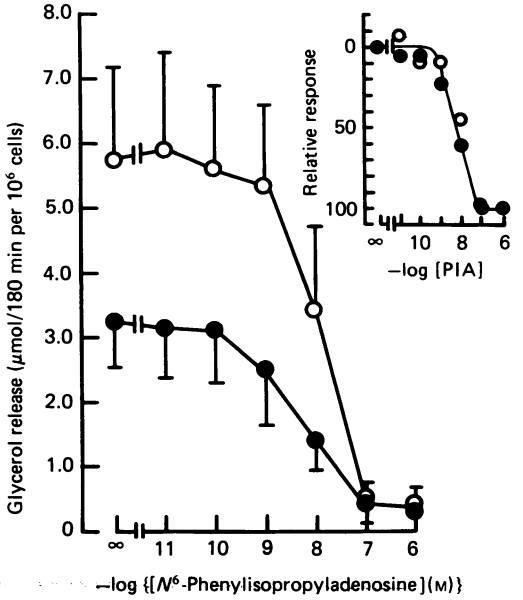

Fig. 3. Inhibition of non-stimulated lipolysis by N^6-phenylisopropyladenosine in the presence of adenosine deaminase

Values are means ± S.E.M. for nine experiments for each condition carried out in duplicate in the same subjects. The medium contained 1.6 μg of adenosine deaminase/ml, corresponding to 320 munits/ml. ●, Before energy restriction; ○, after 28 days of energy restriction. Relative responses shown in the insert are expressed as a percentage of the maximal response. Abbreviation: PIA, N^6-phenylisopropyladenosine.

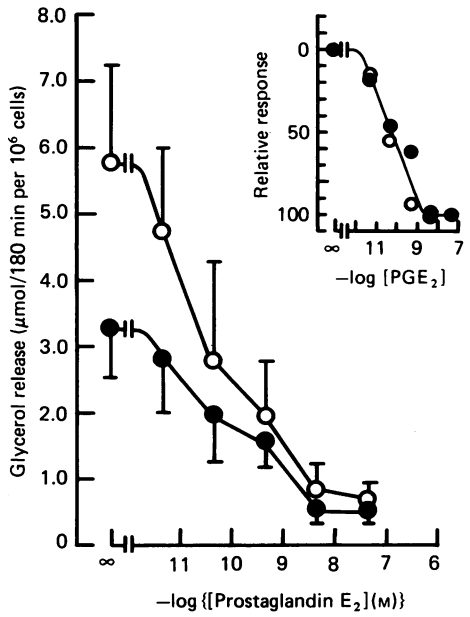

Fig. 4. Dose–response curves for prostaglandin E_2 before and during energy restriction

Values are means ± S.E.M. for nine experiments carried out in the same subjects as, and under experimental conditions identical with, those shown in Fig. 3. For symbols and explanation of insert, see legend to Fig. 3. Abbreviation: PGE_2, prostaglandin E_2.

comparable with those seen during starvation in the absence of adenosine deaminase. However, lipolysis by starved fat-cells was also increased. The increment of glycerol release caused by adenosine deaminase averaged 2 μmol/180 min per 10^6 cells before starvation and 2.2 μmol/180 min per 10^6 cells during starvation, indicating that even extremely dilute suspensions of human fat-cells (10000–20000 cells/ml) produce adenosine in amounts sufficient to depress basal glycerol release under both conditions. From measurements of the maximal activities of enzymes involved in adenosine metabolism, Newsholme *et al.* (1985) concluded that changes of adenosine concentration within adipose tissue are physiologically important during starvation in the rat. Because removal of endogenous adenosine was associated with similar increments of glycerol release before and during starvation, the present findings could suggest that adenosine production and degradation are not substantially altered by starvation. However, the definite answer to this question will depend on direct measurements of adenosine in adipose tissue before and during starvation.

In addition to a possible change in steady-state concentrations of adenosine, starvation has been shown to be associated with decreasing sensitivity of rat adipocytes to the A_1-site-selective adenosine analogue N^6-phenylisopropyladenosine (Chohan *et al.*, 1984). We therefore studied the effects of various concentrations of N^6-phenylisopropyladenosine before and during starvation in the presence of adenosine deaminase (1.6 μg/ml). In contrast with the rat (Chohan *et al.*, 1984), we were unable to detect any changes in sensitivity of human fat-cell lipolysis to N^6-phenylisopropyladenosine (Fig. 3). The relative responses to the adenosine analogue before and during starvation were almost exactly superimposable under these conditions (Fig. 3 insert). Half-maximal effects occurred at approx. 10 nM before and during starvation.

In view of these latter findings, it appeared possible that endogenous inhibitors other than adenosine are more important during dietary adaptation in human adipose tissue. Therefore the dose–response relationships of prostaglandin E_2 were also investigated (Fig. 4). Similarly to N^6-phenylisopropyladenosine, maximally effective concentrations of prostaglandin E_2 almost completely depressed non-stimulated rates of lipolysis in the presence of adenosine deaminase. In addition, the relative response to prostaglandin E_2 was unchanged by starvation (Fig. 4 insert). Half-maximal effects were observed at 50 pM-prostaglandin E_2 under both conditions. This value is considerably lower than that previously observed by us (Kather & Simon, 1981) and by others (Richelson *et al.*, 1984) in surgical subjects. However, prostaglandin sensitivity was assessed in the presence of isoprenaline in both studies. Increasing concentrations of isoprenaline have effects on sensitivity and responsiveness to prostaglandin E_2 similar to those shown for N^6-phenylisopropyladenosine in Fig. 2 (results not shown). It is therefore not surprising that relatively high concentrations of prostaglandin E_2 were needed in these latter studies to obtain half-maximal effects.

In human fat-cells, starvation is not only associated with an increase of basal lipolysis. Concomitantly, the lipolytic effects of adrenaline and noradrenaline are impaired or even reversed (Kjellberg & Östman, 1971;

Berlan *et al.*, 1981; Östman *et al.*, 1984; Kather *et al.*, 1985*a*). Both effects can be mimicked by removal of endogenous adenosine (Kather *et al.*, 1985*a*). These findings led us to conclude that the changes in basal lipolytic rate and responsiveness to catecholamines induced by starvation are mainly due to relief of endogenous inhibitory influences (Kather *et al.*, 1985*a*). The results of the present studies show that the increase in non-stimulated lipolysis seen in human adipocytes during starvation cannot be explained by a decrease in sensitivity or responsiveness to prostaglandin E_2. In addition, if the effects of N^6-phenylisopropyladenosine resemble those of the parent nucleoside, the starvation-induced increase in basal lipolysis is also unrelated to changes in adenosine action. In contrast with the present findings with human fat-cells, Chohan *et al.* (1984) have shown that starvation is associated with decreasing sensitivity of adipocytes to N^6-phenylisopropyladenosine in the rat. However, direct comparisons are difficult. In the present studies the effects of N^6-phenylisopropyl-adenosine were assessed in the absence of a stimulant, whereas in the study of Chohan *et al.* (1984) the effects of the adenosine analogue were examined in the presence of noradrenaline which stimulates lipolysis. It therefore remains doubtful at present whether the differences between our results in humans and those of Chohan *et al.* (1984) in the rat reflect differences between species or are caused by different experimental conditions.

By using pertussis toxin, which blocks the transfer of inhibitory information to adenylate cyclase (Ui *et al.*, 1984), we recently showed that human fat-cell lipolysis proceeds at near-maximal rates upon relief of endogenous inhibition (Kather *et al.*, 1985*b*). In our opinion, it is therefore still most probable that attenuation of inhibitory influences is important for the increase in basal lipolysis seen during starvation. Although other regulators whose structure is not yet known might also be involved (Smith *et al.*, 1976), direct measurements of the concentrations of known inhibitory regulators such as adenosine or prostaglandins within adipose tissue before and during starvation would be of great value in further clarifying the role that this class of modulators might play during dietary adaptation in humans.

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