EFFECTS OF LIPOLYTIC AND ANTI-LIPOLYTIC HORMONES ON PERFUSED RAT FAT CELLS

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ABSTRACT

The effects of various lipolytic and anti-lipolytic hormones on isolated rat epididymal fat cells were studied in a perfusion system. Glycerol release was stimulated rapidly by infusion of 2 ng/ml of ACTH₁₋₂₄, 0.52 mIU/ml of rat ACTH₁₋₃₉, 0.1 μ M glucagon, 0.25 μ g/ml of adrenaline, or 10 mIU/ml of TSH. The stimulated lipolysis decreased rapidly when infusion of the hormones was stopped. However, the stimulation induced by ACTH₁₋₂₄ at a high concentration (2 μ g/ml) persisted for a longer period after the infusion was stopped, while the ACTH₁₋₂₄ concentration dropped rapidly in the cell chamber. The anti-lipolytic effect of insulin at high concentration (2.43 mIU/ml) also persisted for a while, but that of insulin at low concentration (49 μ IU/ml) was rapidly reversed. It is thus concluded that the binding of the lipolytic and anti-lipolytic hormones to fat cell receptors at physiological concentrations, as judged by the lipolytic responses, was not tenacious.

A system for perfusion of isolated rat fat cells has been developed to study the kinetics of the lipolytic and anti-lipolytic actions of various hormones (references 2, 3, 13). This system overcomes the inhibitory effects of free fatty acids produced by lipolysis (5, 6, 12) and of adenosine released from the cells during lipolysis (11, 18) and thus the cells show high sensitivity and rapid responses to infused adrenaline (3, 15), thyrotropin (TSH) (15), ACTH₁₋₃₉ (2, 14) and insulin (1, 21, 23). In contrast to the actions of other lipolytic hormones, the lipolysis induced by ACTH₁₋₃₉ was reported to remain at the stimulated level for at least 30 min after termina-

tion of ACTH₁₋₃₉ infusion (2, 14), during the rapid decrease in its concentration in the medium, suggesting that ACTH₁₋₃₉ bound tenaciously to the receptors on fat cells even after termination of ACTH infusion, or that the action of ACTH₁₋₃₉ was mediated through a distinct time-requiring step. However, the concentration of ACTH₁₋₃₉ employed in these experiments was extremely high (0.1 U/ml) compared with the normal level in rat plasma, which was reported to be less than 10 ng/ml, even in adrenalectomized animals (10, 19). Thus experiments were needed on the lipolytic response of fat cells to a lower ACTH concentration.

Like the action of ACTH, the anti-lipolytic action of insulin also persists for a long time after termination of insulin infusion (1, 21). This finding was explained by proposing that insulin was bound to the cells tenaciously even after its concentration in the medium was reduced, or

Abbreviations: TSH, thyrotropin; ACTH, adrenocorticotrophic hormone; BSA, bovine serum albumin; Tris, tris(hydroxymethyl) aminomethane; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; KRB, Krebs-Ringer bicarbonate buffer

that the effect of insulin persisted in the cells after insulin had dissociated or been released again from the cells.

The present work was undertaken to examine the interactions of ACTH and insulin in lower concentrations with their receptors on the cells using a perfusion system of isolated rat fat cells.

MATERIALS AND METHODS

Adrenaline and synthetic ACTH₁₋₂₄ were purchased from Daiichi Pharmaceutical Co., Tokyo. ACTH₁₋₃₉ (control preparation isolated from rats, 0.52 IU/mg) was kindly supplied by the National Pituitary Agency of DIAMDD, MD. Bovine pituitary thyrotropic hormone (TSH) and bovine pancreatic insulin (24.1 IU/mg) and porcine/bovine pancreatic glucagon were obtained from Sigma Chemical Co., St. Louis. Collagenase from *Clostridium histolyticum* 40BO27 was obtained from Worthington Biochemical Corp., NJ. Bovine serum albumin (Fraction V) was purchased from Armour Pharmaceutical Co., AZ and dialyzed against redistilled water before use. Male Wistar-King rats weighing 120–140 g were purchased from Clea Japan Inc., Osaka. Glycerokinase and 3-glycerophosphate dehydrogenase were obtained from Boehringer-Mannheim GmbH, W. Germany. A jacketed chromatography column (1.6 × 15 cm) was purchased from Amicon Co. Dimethylpolysiloxane was obtained from Fuji System Co., Tokyo. ¹²⁵I-ACTH₁₋₂₄ was obtained from Cea-Ire-Sorin Co., France. ¹²⁵I-Insulin was purchased from Dainabot Radioisotope Lab., Tokyo. All other reagents used were of analytical grade.

Preparation of Fat Cells

Isolated fat cells ($5.5\text{--}6.3 \times 10^6$ cells/g wet weight) were prepared by collagenase treatment from epididymal fat pads of fed male Wistar-King rats by the method of Rodbell (20).

Perfusion of Fat Cells

Fat Cells were perfused as described by Allen *et al.* (3) with minor modifications. Fat cells (0.6 to 1.0 g) were placed in the cell chamber of a glass column siliconized with dimethylpolysiloxane. The fat cells floating solution in the chamber was stirred at 50–100 rpm with a magnetic stirrer during the experiment. Krebs-Ringer bicarbonate buffer or 25 mM Tris-HCl buffer (pH 7.4) containing 0.5 to 1.5% (w/v)

dialyzed bovine serum albumin, 2 mM CaCl₂, 135 mM NaCl, 5 mM KCl, and 1 mM MgCl₂, with or without hormones, was pumped into the column at a rate of 0.83–1.2 ml/min through an infusion tubing (ϕ 1 mm × 123.3 cm with 0.97 ml of dead space) fixed at the top of the column. The perfusate was collected through a tubing (ϕ 1 mm × 70.5 cm with 0.55 ml of a dead space) fixed at a bottom of the column at 1–4 min intervals. A correction was made for the time lags caused by these tubings. Hormone infusion was started by changing the buffer to a solution containing hormone with a two-way tap at the end of the infusion tubing. All the concentrations of hormones and agents given in the text are the final concentrations in the chamber containing the fat cells. The column was kept at 37°C by circulating water in the jacket.

Glycerol Determination

The concentration of glycerol in the perfusate was determined by the method of Chernick (8) and values given in the text are the final concentrations in the perfusate.

Protein Determination

The protein content of isolated fat cells was determined by the Biuret method standardized with bovine serum albumin. The protein content was 13.9 mg/g cells in fat cell preparations.

RESULTS

Stimulation of Lipolysis by Low and High ACTH₁₋₂₄ Concentrations

We compared the time courses of the lipolytic responses to infusion with two different concentrations of ACTH in the cell chamber. The time course of change of ACTH concentration was also estimated with a tracer amount of immunoreactive ¹²⁵I-ACTH₁₋₂₄ (3,700 cpm/μg ACTH). As shown in Fig. 1, when ACTH₁₋₂₄ was infused sequentially at low (2 ng/ml) and high (2 μg/ml) concentrations for 30 min periods with a 30 min interval between the ACTH infusions, the rate of glycerol release increased to a maximum within 30 min in both cases (57 and 113 nmol/min/g cells, respectively) after the start of ACTH₁₋₂₄ infusion. The prolonged effect of ACTH₁₋₂₄ after the termination of its infusion at high concentration (2 μg/ml), as first reported by Katocs *et al.* (14), was observed in the present system; the half time for reversal of stimulated glycerol release ($t_{1/2}$)

Fig. 1 Time courses of concentration change of infused ACTH_{1-24} and the lipolytic response of fat cells. After an 8-min basal period, ACTH_{1-24} (2 ng/ml) was infused at the flow rate of 0.83 ml/min for 30 min. Then a second identical infusion of ACTH_{1-24} (2 $\mu\text{g/ml}$) for 30 min was re-started 30 min after the end of the first infusion. The ACTH_{1-24} concentration change in the cell chamber was measured by ^{125}I -labeled ACTH_{1-24} (3,700 cpm/ μg ACTH). $\circ$, glycerol release; $\bullet$, ACTH_{1-24} concentration

was longer than 60 min. However, the reverse was rapid ($t_{1/2} = 11.3$ min) at low ACTH_{1-24} concentration (2 ng/ml). The time course of change in ACTH_{1-24} at high concentration (2 $\mu\text{g/ml}$) was apparently first order and its half time ($t_{1/2}$) was 3.6 min; but that at low concentration (2 ng/ml) was not detectable because of the low specific activity of ^{125}I - ACTH_{1-24} .

For detailed examination of the concentration change of ACTH_{1-24} in the cell chamber and of the lipolytic response of the fat cells at low ACTH_{1-24} concentrations, ^{125}I - ACTH_{1-24} (0.5 and 2 ng/ml) of higher specific radioactivity (37,500 cpm/ng ACTH_{1-24}) was infused (Fig. 2). In both cases, the concentration of ACTH_{1-24} increased and then decreased in an apparent first order manner. The disappearance rate of ACTH_{1-24} ($t_{1/2} = 3.6$ min) was about the same as in the case of a high concentration of ACTH (2 $\mu\text{g/ml}$) in Fig. 1. In order to increase ACTH_{1-24} concentration rapidly to a given concentration (2 $\mu\text{g/ml}$ in Fig. 1), we added ACTH_{1-24} directly to the cell chamber just before starting ACTH_{1-24} infusion and found that this did not accelerate the lipolytic response further (data not shown). At a lower ACTH_{1-24} concentration (0.5 ng/ml), both stimulation of the lipolytic response and its reversal were

slightly delayed. But a rapid lipolytic response was observed in parallel with change in the ACTH_{1-24} concentration on 2 ng/ml infusion. In both cases, no prolongation of the effect was observed. Modification of the perfusion conditions by prolonging the period of ACTH_{1-24} infusion to 90 min at low concentration (2 ng/ml) or by replacing the Tris buffer as infusion medium with KRB buffer (pH 7.4) did not alter the rate of lipolytic response significantly and the stimulated glycerol release was rapidly reversed ($t_{1/2} = 9.13 \pm 0.24$ min and 8.50 ± 0.29 min, respectively) after removal of ACTH_{1-24} (Table 1). The response to ACTH_{1-39} from rat pituitary was also not delayed ($t_{1/2} = 14.03 \pm 0.29$ min). When pieces of adipose tissue that had not been treated with collagenase were perfused, the rate of glycerol release by infusion of ACTH_{1-24} at a concentration of 5 ng/ml increased with $t_{1/2} = 10.53 \pm 0.74$ min and decreased rapidly after termination of the infusion with $t_{1/2} = 9.83 \pm 0.44$ min (Table 1).

Stimulation of Lipolysis by Various Lipolytic Hormones

As shown in Fig. 3, infusion of adrenaline also stimulated lipolysis in perfused fat cells. The

Fig. 2 Time course of glycerol release stimulated by two sequential ACTH₁₋₂₄ infusions. After an 8-min control period ACTH₁₋₂₄ (0.5 ng/ml) was infused (0.83 ml/min) for 30 min. A second identical infusion of ACTH₁₋₂₄ (2 ng/ml) for 30 min was started at 30 min after the end of the first infusion. ACTH₁₋₂₄ concentration in the cell chamber was determined with ¹²⁵I-labeled ACTH₁₋₂₄ (37,500 cpm/ng ACTH). ○, glycerol release; ●, ACTH₁₋₂₄ concentration

rate of glycerol release increased rapidly after the start of adrenaline infusion and reached a maximum within 30 min. The maximal rate was dose-dependent, being 230 nmol/min/g cells at an adrenaline concentration of 25 μ g/ml and 152 nmol/min/g cells at 0.25 μ g/ml. After stopping the infusion (0.25 μ g/ml), the stimulated glycerol release by adrenaline was reversed rapidly ($t_{1/2}$ =15 min). This result was consistent with that of Katocs *et al.* (15). At the higher concentration (25 μ g/ml), the stimulated

rate persisted for about 15 min after termination of the infusion and then decreased ($t_{1/2}$ =30 min). The half time for disappearance of adrenaline after stopping infusion, which was measured with a trace amount of [¹⁴C]-labeled adrenaline (4,000 cpm/ μ g adrenaline), was 3.6 min in both cases in Fig. 3. The prolongation of the effect of adrenaline after stopping its infusion at high concentration was not so marked as with ACTH (Fig. 1).

Another lipolytic hormone, bovine TSH, was

Table 1 Glycerol Release by Various Lipolytic Hormones

Lipolytic hormone	$t_{1/2}$ for increase in lipolysis (min)	$t_{1/2}$ for decrease of stimulated lipolysis (min)
ACTH ₁₋₂₄ (2 μ g/ml)	6.53 ± 0.27	49.30 ± 2.96
(2 ng/ml)	12.40 ± 2.42	9.63 ± 1.73
(2 ng/ml) ¹	11.03 ± 1.12	8.50 ± 0.29
(5 ng/ml) ²	10.53 ± 0.74	9.83 ± 0.44
(2 ng/ml) ³	8.93 ± 0.35	9.13 ± 0.24
ACTH ₁₋₃₉ (0.52 mIU/ml) ⁴	10.60 ± 0.98	14.03 ± 0.29
Glucagon (10 ⁻⁷ M)	7.30 ± 0.40	8.73 ± 0.93
TSH (10 mIU/ml)	9.60 ± 1.10	11.40 ± 1.65
Adrenaline (0.25 μ g/ml)	10.80 ± 0.83	18.60 ± 0.87

The data represent the mean $\pm$ SE of three separate experiments.

Notes: ¹KRB was used as buffer solution.

²Fat tissue (1 g) was used instead of fat cells.

³ACTH₁₋₂₄ was infused for 90 min.

⁴Rat ACTH₁₋₃₉ was infused.

Fig. 3 Effect of infused adrenaline on glycerol release from perfused fat cells. After a 10 min control period, 0.25 $\mu\text{g/ml}$ (○) or 25 $\mu\text{g/ml}$ (●) of adrenaline was infused (0.78 ml/min) for 30 min into two separate columns containing 0.65 g fat cells from the same preparation.

infused sequentially at two different concentrations (3 and 30 mIU/ml) at intervals of 30 min and found to stimulate the rate of lipolysis in perfused fat cells (Fig. 4). As in the responses to ACTH₁₋₂₄ (Fig. 2) and adrenaline (Fig. 3), the maximal rate of glycerol release depended on the concentration of TSH infused (45 and 105 nmol/min/g cell at 3 and 30 mIU/ml, respectively). At both concentrations, the rate of glycerol release increased rapidly after initiation of the infusion and it decreased rapidly

on stopping the infusion. Under the experimental conditions, the half time of reversal of lipolysis ($t_{1/2}$) was 14 and 18.5 min with 3 and 30 mIU/ml of TSH, respectively. Thus there was no marked difference in the half time for the reversal at the TSH concentrations tested. This was probably because even at a TSH concentration of 30 mIU/ml, the maximal rate of lipolysis was not attained.

The responses of lipolysis in the cells to initiation and termination of glucagon (10^{-7} M)

Fig. 4 Time course of glycerol release stimulated by two sequential TSH infusions. After a 12 min control period, TSH (3 mIU/ml) was infused (0.84 ml/min) for 28 min. A second identical infusion of TSH (30 mIU/ml) for 30 min was started at 28 min after the end of the first.

Fig. 5 Anti-lipolytic action of insulin on ACTH_{1-24} -stimulated lipolysis in perfused fat cells. After a 10 min control period, ACTH_{1-24} (2 ng/ml) was infused (0.83 ml/min) for 90 min. Thirty minutes after starting ACTH_{1-24} infusion, insulin (49 $\mu\text{IU/ml}$) was concurrently infused for 30 min. The insulin concentration in the cell chamber was determined using ^{125}I -labeled insulin (250 cpm/ μIU). $\circ$, glycerol release; $\bullet$, insulin concentration

infusion were also rapid and comparable to those with other lipolytic hormones (Table 1).

Anti-lipolytic Action of Insulin

The responses of fat cells to the anti-lipolytic action of infused insulin in the presence of various lipolytic hormones were also studied with the perfusion system. As shown in Fig. 5, when insulin (49 $\mu\text{IU/ml}$) was infused 30 min after the start of ACTH_{1-24} (2 ng/ml) infusion, the stimulated rate of glycerol release (115 nmol/min/g cells) was reduced to nearly the basal level (20 nmol/min/g cells). When the infusion of insulin was stopped while that of ACTH_{1-24} was continued, the rate of glycerol release rapidly increased to almost the previous stimulated level (95 nmol/min/g cells). The half time for inhibition by insulin and for its reverse was 9.20 ± 0.61 and 12.40 ± 0.45 min, respectively (Table 2). Under these experimental conditions, increase and decrease in the concentration of insulin in the cell chamber, which was measured with ^{125}I -labeled insulin (250 cpm/ μIU), followed apparently first order curves with a half time of 3.6 min. Under similar perfusion conditions, the effect of insulin on lipolysis induced by TSH was also pronounced

(Fig. 6). Decrease in glycerol release by initiation of insulin infusion and increase in the release by the termination were both rapid ($t_{1/2}$: 9.0 and 12.0 min, respectively) even when insulin was infused at higher concentration (243 $\mu\text{IU/ml}$).

Effect on glycerol Release by Insulin in Rat Fat Cell Perfusion in the Presence of Various Lipolytic Hormones

After a blank perfusion for 10 min, various lipolytic hormones were infused for 90 min into the cell chamber (5 ml) containing 1 g of fat cells (Table 2). Thirty minutes after the start of infusion of lipolytic hormones, various concentrations of insulin were also infused for 30 min. The rates of change in insulin concentration after the start and the end of infusion were the same in each experiment with the same half time ($t_{1/2}$). The lipolytic responses of the fat cells to all the hormones tested were found to be rapidly suppressed with simultaneous infusion of insulin at concentrations of 49 $\mu\text{IU/ml}$ and was again rapidly stimulated on termination of insulin infusion. But when the insulin concentration was raised to 2.43 mIU/ml, TSH-induced lipolysis was suppressed with $t_{1/2} = 6.17 \pm 0.44$

Fig. 6 Anti-lipolytic action of insulin on TSH-stimulated glycerol release from perfused fat cells. After a 9 min control period, TSH (10 mIU/ml) was infused (1.2 ml/min) for 90 min. Thirty minutes after starting TSH infusion, insulin (243 μ IU/ml) was concurrently infused for 30 min.

min, while the response of glycerol release to TSH after termination of insulin infusion was significantly delayed ($t_{1/2} = 19.17 \pm 0.73$ min) (Table 2).

DISCUSSION

The mechanism of hormone action was examined by measuring the initial step of hormone-receptor interaction with specific binding analysis and determination of the affinities of hormones for cell receptors. In those experiments, cells were usually washed with a chilled buffer solution after incubation with hormones to minimize nonspecific binding. On the other hand this procedure might cause alteration of the characteristics of the cells and their receptors (17). A perfusion system was then developed (2, 13) in which the response of the cells to infused

hormones can be observed as a better indicator of the hormone-receptor interaction. Perfusion of fat cells is useful for studying rapid lipolytic responses of the cells to various hormones with high sensitivity, because it avoids the inhibitory effects of fatty acids (5, 6, 12) and adenosine (11, 18), which accumulate during the lipolytic reaction in a fixed volume of incubation medium. Using a perfusion system, glycerol release as a lipolytic response of the fat cells could be detected even when a low concentration of $ACTH_{1-24}$ was used (*cf.* Fig. 2) (10, 19). It was also demonstrated by using a similar method that the rate of release of glycerol, a product of lipolysis, increased rapidly after initiation of $ACTH_{1-39}$ (0.1 U/ml) infusion, but decreased much more slowly after termination of the infusion (2, 14). This delayed response of the cells to the removal of the hormone was shown

Table 2 *Effect on Glycerol Release by Insulin*

Lipolytic hormone		Insulin concentration (μ IU/ml)	$t_{1/2}$ for insulin concentration change (min)	$t_{1/2}$ for decrease of stimulated lipolysis (min)	$t_{1/2}$ for recovery of inhibited lipolysis (min)
TSH	(10 mIU/ml)	486	2.8	7.83 ± 0.73	12.13 ± 0.47
TSH	(10 mIU/ml)	2,430	2.8	6.17 ± 0.44	19.17 ± 0.73
Adrenaline	(150 ng/ml)	49	3.6	9.07 ± 0.53	14.87 ± 0.47
$ACTH_{1-24}$	(2 ng/ml)	49	3.6	9.20 ± 0.61	12.40 ± 0.45
Glucagon	(10^{-7} M)	49	3.6	7.17 ± 0.60	17.10 ± 0.97

The data represent the mean $\pm$ SE of three separate experiments.

to be specific for ACTH and was not seen with other lipolytic hormones, such as adrenaline, thyrotropin (TSH), and glucagon (3). As shown in Fig. 1, the lipolytic response was still pronounced at 2 ng/ml ACTH₁₋₂₄, the level of glycerol release reaching approximately one half of that obtained in the lipolytic response at 2 μ g/ml ACTH₁₋₂₄. Moreover, at the lower ACTH₁₋₂₄ concentration there was no delay in termination of lipolytic stimulation after removal of ACTH₁₋₂₄ (Fig. 2) at a fixed concentration of Ca²⁺ (2 mM). This finding was in contrast to that of Katocs *et al.* (14). This rapid response was not specific for ACTH₁₋₂₄ under the conditions in the experiment shown in Fig. 2, but was also observed in different experimental systems as indicated in Table 1. The marked delay in ending stimulated lipolysis after termination of ACTH₁₋₂₄ infusion at a high concentration (2 μ g/ml) can be explained on the basis of the rate of change in concentration of ACTH₁₋₂₄, as follows. After stopping ACTH₁₋₂₄ perfusion, it was calculated to take about 36.0 min for the ACTH₁₋₂₄ concentration to decrease from 2 μ g/ml to 2 ng/ml, since the half time of the ACTH₁₋₂₄ disappearance (3.6 min) was observed (Figs. 1 and 2) at both ACTH₁₋₂₄ concentrations.

A similar, but less marked, delay was also seen with adrenaline at higher concentration (25 μ g/ml in Fig. 3) and with insulin in its inhibitory effect (Table 2). These effects could also readily be explained, without assuming any special tenacious hormone-receptor interaction, when one considers the time required to reduce the hormone concentration in the medium. The difference in the periods of delay with ACTH₁₋₂₄, adrenaline, and insulin might be due to the different dose-dependency of the respective hormones.

Rapid reverse of the inhibitory action was also observed after prolonged infusion of insulin (60 min) with all concurrently infused lipolytic hormones (Figs. 5 and 6, and Table 2). Recently, using a perfusion system, Solomon *et al.* (22) observed that the anti-lipolytic action of insulin was prolonged after removal of insulin at high concentration, but not at low concentration. From this observation, they concluded that the prolonged effect of insulin was due not simply to passive dissociation of the hormone from the receptor but to some mediating step. However, they did not take into account the dose-dependency of the effect of insulin or the rate of change of insulin concentration in the medium. As shown in our work, if the period

required to decrease the insulin concentration in the cell chamber after the end of its infusion ($t_{1/2}$ = 2.8 min) is taken into account, the apparent delay of about 7 min (*cf.* Table 2, the second data) could readily be explained, since about 9.3 min were required for the insulin concentration (2.43 mIU/ml) to decrease to one tenth (243 μ IU/ml) of its initial level, and even this lower concentration was sufficient to inhibit glycerol release (*cf.* Fig. 6). Almost complete inhibition of stimulated lipolysis was observed at the low concentration of infused insulin and rapid recovery of lipolytic responses to all hormones tested was also observed after removal of insulin.

The prolonged effect of ACTH and insulin on the adipocyte lipolysis was due to the high concentration of those hormones used in the previously reported experiments, but, the long term action of the adipocyte lipolytic response by lipolytic or antilipolytic hormone was not observed when a low hormone level was used. From these results, we conclude that hormonal information related to the lipolytic response is rapidly transferred to the cell. The specificity of hormone-receptor interaction might help to clarify the hormonal regulation of the lipolysis and lipogenesis in the adipocyte. More detailed studies on the hormone effect on fat cells are in progress.

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