INCREASED SENSITIVITY TO EPINEPHRINE OF THE CYCLIC AMP–PROTEIN KINASE SYSTEM IN ADIPOSE TISSUE OF DIABETIC RATS

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1. Introduction

Adipose tissue from streptozotocin-diabetic rats displays an increased sensitivity to the lipolytic action of epinephrine – but not to that of ACTH, glucagon or dibutyryl cyclic AMP – as compared to normal adipose tissue [1]. The question which arose from these findings was whether the receptor-mediated action of catecholamines on the cyclic AMP–protein kinase system, which has been shown to be involved in the stimulation of lipolysis by lipolytic hormones (reviewed [2]), was enhanced in diabetic adipose tissue. Therefore, we studied and compared the responses of cyclic AMP and of the cyclic AMP-dependent protein kinase to epinephrine and ACTH in adipose tissue from normal and diabetic rats. We found that cyclic AMP responses and protein kinase activation are more sensitive to epinephrine, but not to ACTH, in adipose tissue of diabetic rats. Our results are compatible with an increased sensitivity of the catecholamine receptor–adenylate cyclase system of rat adipose tissue in the diabetic state.

2. Materials and methods

2.1. *Animals*

Male Zbz Cara (formerly Osborne-Mendel) rats, 130–160 g, were used for all experiments. They received NAFAG chow (NAFAG no. 890, Gossau, Switzerland) and had free access to drinking water. Diabetic animals were sacrificed 5 days after i.v. administration of 70 mg/kg streptocotocin (Eli Lilly). Their body weight had not increased during that time, their urine contained > 2% glucose (Tes-Tape, Lilly), but no ketone bodies. The blood sugar ranged from 3.5–5.7 g/l. The fat pad weights had decreased from 40–70%.

2.2. *Incubation of adipose tissue*

Pooled adipose tissue (100–200 mg/vial) was preincubated at 37°C for 1 h in 3 ml Krebs-Ringer bicarbonate buffer containing 3 g/100 ml human serum albumin (HSA, Swiss Red Cross, Bern; free fatty acid content from 6–12 mequiv./g, 0.4–0.8 mol FFA/mol albumin) and 11 mmol/l glucose. Hormones or buffer (control) were then added and the incubation was continued for another 10 min.

2.3. *Determination of glycerol release*

Glycerol release from the tissue was measured by the method in [3] as detailed [1]. Hormone-stimulated glycerol production was linear over 30 min incubation following the preincubation period without hormones. Furthermore, the lipolytic rate was linearly related to the tissue wet wt up to 200 mg/3 ml incubation medium.

Stimulation of lipolysis is expressed as % above basal lipolytic rate set at 100% (see fig.1,2 legends).

2.4. *Determination of cyclic AMP and cyclic AMP-dependent protein kinase*

Adipose tissue was filtered over nylon stocking, 10 min after addition of hormone or buffer, rinsed with ice-cold saline, rapidly blotted on filter paper and homogenized in a Dounce all-glass homogenizer in 7 vol. ice-cold NaCl (0.5 mol/l), pH 6.5, containing 10 mmol/l EDTA, 5 mmol/l theophylline and

10 mmol/l NaF. The homogenate was centrifuged at 35 000 × *g* for 10 min at 4°C. The fat cake was removed with a spatula, and the infranatant used (i) for the determination of cyclic AMP; (ii) for the determination of protein kinase activity.

(i) Infranatant, 1 ml, was deproteinized with 0.2 ml 30% perchloric acid and the precipitate removed by centrifugation for 20 min at 4°C and 3000 × *g*. To 1 ml deproteinized supernate 0.3 ml 5 mol/l $KHCO_3$ solution was added. After 30 min at 0°C the $KClO_4$ precipitate was centrifuged off.

Cyclic AMP was determined in this last neutral supernate by the method in [4]: To 200 μl supernate or of different cyclic AMP dilutions containing from 0–10 pmol cyclic AMP (for standard curve; the dilutions were made with the homogenizing buffer which was previously 'deproteinized' and neutralized as described above) was added 50 μl (~33 nCi; ~1.1 pmol) of a cyclic [^{3}H]AMP solution (in 50 mmol/l potassium phosphate buffer, pH 6.8) and 50 μl 'reconstituted' protein kinase (Sigma no. P-5511; 1 mg lyophilized powder reconstituted with 12 ml H_2O). After 90 min incubation in an ice-bath, 2 ml above potassium phosphate buffer was added, and the mixture was filtered under soft vacuum on Sartorius filters (SM 11306, diam. 25 mm, pore size 0.45 μm). The filters were washed 4 times with 2 ml each of the same buffer, dried, dissolved in 2 ml Cellosolve (Packard) and counted in 10 ml Instagel (Packard) in a liquid scintillation counter.

(ii) Protein kinase was determined by the method in [5,6]: 25 μl infranatant was added to 75 μl 12.5 mmol/l potassium phosphate buffer, pH 6.8, containing per liter: 37 mmol NaF, 30 g histone (type II A from calf thymus, Sigma), 4.5 mmol magnesium acetate, 0.5 mmol [γ-^{32}P]-ATP and, when present, 2 μmol cyclic AMP (for the determination of total protein kinase activity). Incubation was carried out in triplicates for 5 min at 30°C. Then, 50 μl reaction mixture was pipetted on 2 × 2 cm filters (Whatman 31 ET), which were rapidly immersed in a reservoir of 1 liter of ice-cold 10% trichloroacetic acid (TCA). After 2 h gentle stirring the filters were washed for 1 h with the same amount of trichloroacetic acid (TCA) at 0°C, and after a second wash with TCA at room temperature they were transferred to a bath containing absolute ethanol for 5 min. At last, the filters were washed in ether, dried and counted in 7.5 ml Instagel in a liquid scintillation counter. The protein kinase reaction was linear over 10 min with protein concentrations $\leqslant$ 120 μg in 25 μl added infranatant.

The kinetics of the cyclic AMP and protein kinase responses were different for epinephrine and ACTH: Peak levels were reached 5 min after stimulation with ACTH. With epinephrine both indices showed a further rise between 5 and 10 min, whereas they remained on a plateau with ACTH. After 10 min cyclic AMP levels and protein kinase activities started to decline with a half-life of ~10 min (epinephrine) and ~20 min (ACTH) (results not shown).

Protein in the homogenates was determined by Lowry's method [7].

$ACTH_{1-24}$ (Synacthen) was from Ciba-Geigy, Basel, L-epinephrine–HCl from Fluka, Buchs, Switzerland. [γ-^{32}P]ATP and cyclic [^{3}H]AMP were obtained from the Radiochemical Centre, Amersham.

3. Results and discussion

According to the dose–response curves shown in fig.1, the epinephrine concentrations required for half-maximal responses of cyclic AMP production and protein kinase activation are lower in diabetic (180 ng/ml and 50 ng/ml) than in normal adipose tissue (500 ng/ml and 180 ng/ml). In both tissues higher hormone concentrations are needed for half-maximal cyclic AMP responses than for half-maximal activation of protein kinase (see also below for ACTH). Basal cyclic AMP levels (per mg protein) and protein kinase activity ratios are not significantly different for the two tissues ($p > 0.05$): cyclic AMP (mean ± SEM) 13.2 ± 0.6 (n = 24) and 15.8 ± 0.95 (n = 21) pmol/mg protein for normal and diabetic tissue, respectively; protein kinase activity ratios (–/+ 2 × 10^{-6} mol/l cyclic AMP): 0.216 ± 0.013 and 0.234 ± 0.019. If cyclic AMP levels are expressed per mg tissue wet wt they are significantly higher ($p < 0.001$) in diabetic (0.30 ± 0.02 pmol/mg) than in normal tissue (0.19 ± 0.01 pmol/mg) due to the higher protein concentration of diabetic fat pads (20.8 ± 2.1 compared to 14.5 ± 1.1 mg/g tissue wet wt).

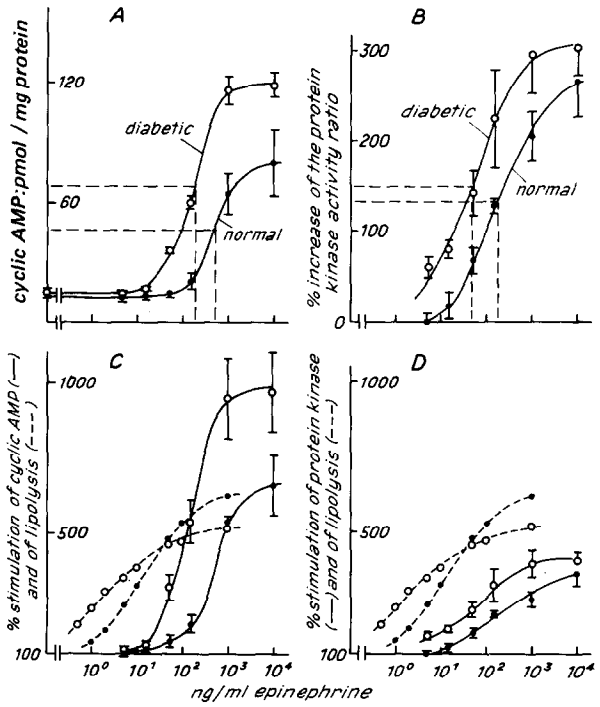

Fig.1. Dose–response curves of epinephrine-stimulated cyclic AMP (A,C) and protein kinase activation (B,D) in adipose tissue of normal (closed symbols) and diabetic rats (open symbols). Different amounts of epinephrine were added after 1 h preincubation. 10 min after hormone addition the tissue was filtered over nylon stocking, rinsed with ice-cold saline, blotted, homogenized and centrifuged as described in section 2. Cyclic AMP and protein kinase activity (in the absence and presence of 2×10^{-6} mol/l cyclic AMP: –/+ ratio) were determined as detailed in the text. Basal cyclic AMP levels and protein kinase activity ratios are listed in results and discussion. Maximal protein kinase activity ratios at 10 μg/ml epinephrine were 0.758 ± 0.046 (n = 3) for normal and 0.755 ± 0.01 (n = 3) for diabetic tissue ($p > 0.05$). In C and D the increase in cyclic AMP and protein kinase activity have been plotted as % basal levels (set at 100%) and compared with % stimation of lipolysis. Basal lipolytic rates were 0.44 ± 0.02 μmol glycerol/pad × h (n = 22) for normal pads and 0.42 ± 0.04 μmol glycerol/pad × h (n = 21) for diabetic pads. Each point is the mean of 3 different experiments. Bars give the SEM. Dotted lines indicate half-maximal stimulation.

In contrast to epinephrine stimulation, half-maximal responses in cyclic AMP production and protein kinase activation are elicited by similar ACTH concentrations in diabetic and normal adipose tissue (at 50 ng/ml for cyclic AMP and at 30–40 ng/ml for protein kinase; fig.2). These results fit our earlier findings on epinephrine- and ACTH-stimulated lipolysis: the lipolytic sensitivity of diabetic adipose tissue towards epinephrine is increased ([1] and fig.1C,D), whereas that towards ACTH is even somewhat lower than in normal tissue ([1] and fig.2C,D). Together with the observation that adenylate cyclase in fat-cell ghosts from diabetic rats is also more sensitive to epinephrine than in fat-cell ghosts from normal animals ([8], in preparation) these findings corroborate the previous concept of an increased sensitivity of the catecholamine receptor–adenylate

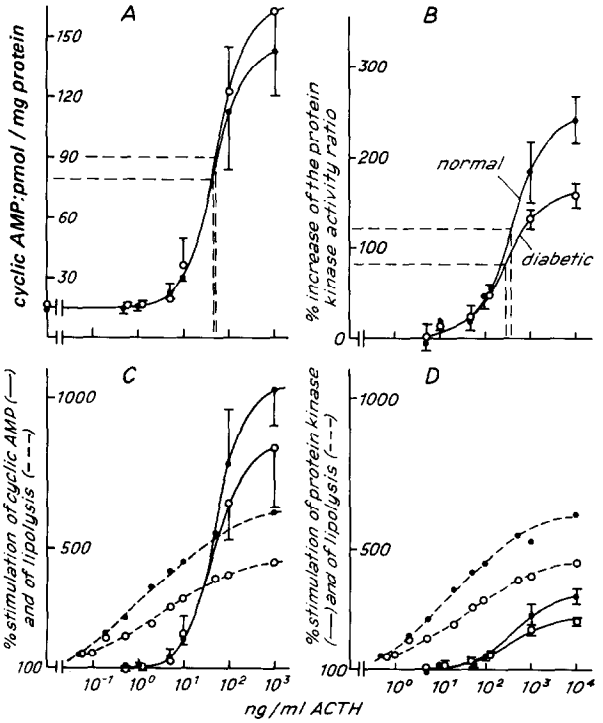

Fig.2. Dose–response curves of ACTH-stimulated cyclic AMP (A,C) and protein kinase activation (B,D) in adipose tissue of normal (closed symbols) and diabetic rats (open symbols). The experimental procedure was the same as in fig.1. In C and D the increase of cyclic AMP and protein kinase activation have been plotted as % basal levels and compared with % stimulation of lipolysis. Each point represents the mean of 3 different experiments, bars give the SEM. Dotted lines indicate half-maximal stimulation. Maximal protein kinase activity ratios at 1 μg/ml ACTH were 0.664 ± 0.04 (n = 4) for normal and 0.715 ± 0.088 (n = 3) for diabetic tissue ($p > 0.05$).

cyclase system of rat adipose tissue in the diabetic state [1].

So far, no conclusive evidence has been obtained that catecholamine uptake into adrenergic nerve endings (reviewed [9]) plays a significant role in causing hypersensitivity of diabetic adipose tissue: Isolated fat cells prepared by collagenase digestion are devoid of nerve endings. Nevertheless, adipocytes from diabetic rats display a higher lipolytic sensitivity to epinephrine than normal fat cells (in preparation). Furthermore, inhibitors of catecholamine uptake, such as cocaine and ouabaine [9,10], do not potentiate the lipolytic action of epinephrine on rat adipose tissue (unpublished observation).

As can be seen from fig.1C,D and fig.2C,D, lipolysis is already stimulated to a considerable extent by epinephrine and ACTH concentrations which do not yet cause a significant increase in tissue cyclic AMP or protein kinase activation. One possible explanation for this is that overall cyclic AMP levels and protein kinase activities may not adequately reflect the true degree of activation of the system. Another explanation, which is based on the comparison between the lipolytic, cyclic AMP and protein kinase responses to epinephrine and ACTH in whole adipose tissue and isolated fat cells [11], would be that at low hormone concentrations lipolysis is mainly stimulated via a cyclic AMP-independent mechanism. Evidence for partly cyclic AMP-independent ACTH-stimulated lipolysis has been obtained [12]. In addition, cyclic AMP-independent hormonal effects have also been found in the perfused rat liver [13] and in isolated rat liver cells [14,15].

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References

[1] Zapf, J., Feuerlein, D., Waldvogel, M. and Froesch, E. R. (1975) Diabetologia 11, 509–516.
[2] Fain, J. N. (1973) Pharmacol. Rev. 25, 67–118.
[3] Eggstein, M. and Kreutz, F. H. (1966) Klin. Wschr. 44, 262–267.
[4] Gilman, A. (1970) Proc. Natl. Acad. Sci. USA 67, 305–312.
[5] Corbin, J. D., Soderling, T. R. and Park, C. R. (1973) J. Biol. Chem. 248, 1813–1821.
[6] Corbin, J. D. and Reimann, E. M. (1974) Methods Enzymol. 18 C, 287–289.
[7] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[8] Zumstein, P., Zapf, J. and Froesch, E. R. (1976) Diabetologia 12, 428.
[9] Trendelenburg, U. (1967) Pharmacol. Rev. 18, 629–640.
[10] Dengler, H. J., Spiegel, H. E. and Titus, E. O. (1961) Nature 181, 816–817.
[11] Zapf, J., Waldvogel, M., Zumstein, P. and Froesch, E. R. (1978) Experientia 34, 29.
[12] Lang, U. and Schwyzer, R. (1972) FEBS Lett. 21, 91–94.
[13] Jakob, A. and Diem, S. (1975) Biochim. Biophys. Acta 404, 57–66.
[14] Exton, J. H., Cherrington, A. D., Hutson, N. J., Assimacopoulos-Jeannet, F. D. and Blackmore, P. F. (1977) Proc. 11th FEBS Meet. Copenhagen, symp. A 1-9, L 2.
[15] Tolbert, M. E. M., Butcher, F. R. and Fain, J. N. (1973) J. Biol. Chem. 248, 5686–5692.