Reversible reduction of insulin receptor affinity by ATP depletion in rat adipocytes

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The effects of the metabolic inhibitor NaN_3 on insulin receptors in isolated rat fat-cells were investigated. The agent reduced insulin binding in parallel to a decrease of the ATP content of cells. Both effects were observed in the same concentration range of NaN_3 , and were fully reversible. According to the binding curves the affinity rather than the number of receptors was reduced. Kinetic experiments revealed an increased dissociation rate of the insulin–receptor complex. The effects outlasted cell disruption, since the receptor affinity was still lowered in plasma membranes obtained from NaN_3 -treated cells. Thus an inhibition of insulin internalization could not account for the observed effects. It is suggested that the observed ATP-dependence of insulin receptor affinity reflects a reversible structural alteration of the receptor, or of some closely related membrane protein.

The action of insulin is initiated by binding of the hormone to a specific receptor in the plasma membrane. Number as well as affinity of these receptors are variable, and may therefore contribute to the regulation of insulin sensitivity in target tissues. Several findings have suggested that intracellular metabolic events can modify insulin binding (Caro & Amatruda, 1980; Filetti *et al.*, 1981; Steinfelder & Joost, 1982). A regulatory role of energy metabolism in insulin binding was first proposed by Yu & Gould (1977), who reported that anoxia decreased insulin binding and insulin action in isolated soleus muscle of the rat. Similar effects of metabolic inhibitors have since been observed in hepatocytes (Draznin *et al.*, 1980) and in adipocytes (Häring *et al.*, 1981; Joost & Steinfelder, 1983).

The coupling process between insulin receptors and insulin action is ATP-dependent, whereas the effects of insulin, once initiated, outlast ATP depletion of cells (Häring *et al.*, 1981). When occupied, insulin receptors seem to undergo several alterations that might be involved in the coupling process. The receptors are phosphorylated (Kasuga *et al.*, 1982), attain a higher apparent affinity (Corin & Donner, 1982) and are progressively internalized (Gordon *et al.*, 1978). These events presumably

require ATP, and they might be related to the ATP-dependent step of the coupling process. Therefore, the present study was designed to characterize the ATP-dependence of insulin binding by answering the following questions. First, does a metabolic inhibitor (NaN_3) modify the number or the affinity of receptors, hence altering the receptor turnover or the receptor structure? Secondly, are the effects reversible and are they thus of some significance in the normal regulation of receptor binding? Thirdly, are the effects retained in plasma-membrane preparations of pretreated cells, or do they disappear when cells are disrupted, because the effects largely reflect inhibition of internalization of the hormone?

Experimental

Materials

Male Wistar rats (180–275 g) bred in our Institute were used throughout. The animals had free access to food (standard pellet diet) and water. Crystalline porcine insulin was obtained as a gift from Hoechst A.-G. (Frankfurt, Germany). ^{125}I -Insulin was purchased from Behringwerke A.-G. (Frankfurt, Germany), bovine serum albumin was from Serva (Heidelberg, Germany), Hepes was from Sigma (St. Louis, MO, U.S.A.) and crude bacterial collagenase (EC 3.4.24.3; type IV) was from Worthington (Freehold, NJ, U.S.A.). The reagents for the ATP assay were supplied by Boehringer (Mannheim, Germany). All other reagents (ana-

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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lytical grade) were obtained from Merck A.-G. (Darmstadt, Germany).

Isolation and incubation of adipocytes

Isolated adipocytes were prepared by the method of Rodbell (1964) from the epididymal fat-pads of male Wistar rats (180–275 g) fed *ad libitum*, with modifications described previously (Joost & Steinfelder, 1982). The cells were washed and incubated in Krebs–Ringer phosphate buffer (Fain, 1973) containing 4% bovine serum albumin.

Insulin binding

Binding of insulin to isolated fat-cells was assessed as previously described in detail (Joost *et al.*, 1982). Cells were incubated in the presence of 0.11 nM labelled insulin and unlabelled insulin as indicated. The incubation was terminated by the oil flotation method (Gliemann *et al.*, 1972), and total cell-associated radioactivity was determined. All values were corrected for non-specific binding, which was defined as the amount of radioactivity not displaceable by 10 μ M unlabelled insulin.

Plasma membranes (approx. 75 μ g of protein/sample) were incubated for 60 min in the presence of 0.22 nM labelled insulin and the desired concentrations of unlabelled insulin. The incubation was stopped by centrifugation (10000 g, 2 min), the supernatant was removed and the surface of the pellet was washed with ice-cold buffer. The tip of the vial was cut off, and total bound radioactivity was determined. All values were corrected for non-specific binding as assessed in the presence of 10 μ M unlabelled insulin.

Preparation of plasma membranes

After pre-incubation, the cells were washed five times with calcium- and albumin-free Krebs–Ringer Hepes buffer containing (mM): Na⁺, 143; K⁺, 5.9; Mg²⁺, 1.2; HPO₄²⁻, 1.2; HCO₃⁻, 1; Hepes, 10. The cell suspension was pressed and sucked five times through a stainless steel cannula (0.65 mm in diameter) with the aid of a plastic syringe. In our hands this method of cell disruption was superior to that achieved by vortex-mixing or by high-pressure nitrogen decompression. The homogenate was centrifuged for 30 s at 4000 g (20°C) to remove the lipids, and the infranant and a small pellet were aspirated. Nuclei were removed by centrifugation at 600 g for 20 min (4°C). The resulting supernatant was layered on a discontinuous sucrose gradient (27 and 32%, w/v) and spun at 60000 g (rotor SW 27, 20000 rev./min) in a Beckman ultracentrifuge for 30 min. The plasma membranes, which formed a band at the interface between the two sucrose solutions, were removed, diluted with buffer and sedimented (60000 g, 30 min). Purity was assessed by determination of succinate dehydrogenase and

5'-nucleotidase. The membrane fraction contained about 30% of the total 5'-nucleotidase activity enriched 6-fold in specific activity. According to the succinate dehydrogenase activity, mitochondrial contamination was about 10%. The resuspended membranes (approx. 1 mg of protein/ml) were frozen and stored in liquid N₂ until used for binding experiments.

Assay of protein, ATP and enzyme activities

Protein was assayed by the method of Bradford (1976), with bovine serum albumin as standard. ATP was determined as described by Williamson & Corkey (1969). The assay is based on the fluorimetric determination of NADPH generated in a hexokinase/glucose 6-phosphate dehydrogenase coupled reaction. Succinate dehydrogenase (EC 1.3.99.1) was determined with 2,6-dichloroindophenol by the method of Bachmann *et al.* (1966). 5'-Nucleotidase (EC 3.1.3.5) was assayed as the conversion of [2-³H]AMP into [³H]adenosine by the method of Newby *et al.* (1975).

Results

When fat-cells were incubated for 15 min in the presence of NaN₃, the cellular ATP content decreased in a concentration-dependent pattern (Fig. 1). In parallel, specific insulin binding was reduced. The concentrations of NaN₃ that produced a half-maximal effect were similar, whereas the maximal effects differed: insulin binding could be lowered by 40%, whereas ATP content was reduced by 90% by high NaN₃ concentrations. As can be seen in Table 1, both effects were fully reversed when ATP-depleted cells were incubated in the presence of glucose and adenosine.

A displacement experiment (Fig. 2) revealed that ATP-depletion had induced a rightward shift of the binding curve. The concentration of unlabelled insulin that displaced 50% of tracer insulin was approximately doubled from 2.63 nM in the control experiments to 5.25 nM in ATP-depleted cells. Thus the overall apparent affinity of insulin receptors was reduced in the ATP-depleted cells.

Since a reduced receptor affinity should be accompanied by alterations of the association or dissociation rate, kinetic experiments were performed. Alterations of the association rate could not account for the observed effect, since insulin binding was essentially the same in ATP-depleted cells ($0.657 \pm 0.1\%/10^6$ cells per ml) as in control cells (0.662 ± 0.12) after 5 min. At this incubation time binding of insulin is largely governed by the association rate, since dissociation of the insulin–receptor complex is considerably slower. At equilibrium (90 min), however, a significant difference was observed (1.41 ± 0.2 compared with $2.65 \pm 0.4\%/10^6$

Table 1. *Reversibility of the effects of NaN₃ on insulin binding and ATP content of adipocytes*

Isolated fat cells were incubated for 15 min at 37°C in the presence of NaN₃ (4 mM) or buffer (controls), and were washed twice with buffer. In separate experiments it was confirmed that this treatment was sufficient to lower the ATP content of cells by at least 80%. The second incubation was carried out for 30 min at 37°C in the presence of buffer or glucose (10 mM) and adenosine (0.1 mM) as indicated. Insulin binding was assayed thereafter at 22°C, and ATP was determined in parallel samples incubated and stopped according to the same schedule. Data are means ± s.e.m. of six experiments, and were tested for significance with the D₁-test of Wilcoxon and Wilcox. Abbreviation used: n.s., not significant.

		ATP depletion		Restoration of ATP levels		Control Buffer
	First incubation ...	NaN ₃		NaN ₃		
	Second incubation ...	NaN ₃		Glucose + adenosine		Glucose + adenosine
ATP (nmol/10 ⁶ cells)		1.82 ± 0.3	<i>P</i> < 0.025	18.05 ± 1.64	n.s.	16.97 ± 1.34
Insulin binding (%/10 ⁶ cells per ml)		1.97 ± 0.4	<i>P</i> < 0.025	4.94 ± 0.36	n.s.	5.07 ± 0.76

Fig. 1. *Effects of NaN₃ on insulin binding (○) and ATP content (●) of isolated rat fat-cells*

Adipocytes [(0.6–1.2) × 10⁶ cells/ml] were incubated in the presence of NaN₃ as indicated for 15 min at 37°C, and insulin binding was determined after equilibration at 20°C for 90 min. ATP was assayed in parallel samples that had been incubated and stopped according to the same schedule. Data are means ± s.e.m. of five experiments.

cells per ml). As can be seen from Fig. 3, the dissociation rate of the insulin–receptor complex was significantly increased in ATP-depleted cells. In the NaN₃-treated cells 50% of the bound hormone dissociated after 14 min compared with 32 min in the control cells. Therefore the lowered affinity of insulin receptors in ATP-depleted adipocytes is due to an accelerated dissociation of bound hormone.

When ATP-depleted cells were homogenized and plasma membranes were prepared, insulin binding to the membranes was still significantly lower

Fig. 2. *Displacement of ¹²⁵I-insulin by unlabelled insulin in NaN₃-treated (1.5 mM, ○) and control cells (●)*

The experimental set-up was identical with that of Fig. 1. Data are means ± s.e.m. of six experiments. Arrows indicate the insulin concentrations that displaced 50% of the tracer insulin. The inset shows a Scatchard plot of the data.

(11.9 ± 1.2 fmol/mg of protein) than in membranes of untreated cells (22.6 ± 2.2 fmol/mg of protein; n = 6; P < 0.025). Protein content (23 μg ± 6 μg/10⁶ cells compared with 20 ± 5 μg/10⁶ cells) and 5'-nucleotidase activity (21.0 ± 8.6% compared with 21.4 ± 7%) were not significantly altered by the NaN₃ treatment.

In a second series a displacement experiment was carried out on pooled plasma membranes of ATP-depleted cells obtained from a total of 12 animals (Fig. 4). In this series the decrease of ¹²⁵I-insulin binding in the absence of unlabelled insulin was lower (25%) than that of the former experiment (50%). The slope of the Scatchard plot (inset) was reduced by pretreatment of cells with NaN₃, indicating that the affinity rather than the number of receptors was reduced by ATP depletion.

Fig. 3. Dissociation of bound insulin from control (●) and ATP-depleted (4 mM- NaN_3 treatment, ○) adipocytes. Binding was equilibrated in the presence of 0.11 nM- ^{125}I -insulin for 90 min at 22°C. To start dissociation, the cell suspension was diluted 80-fold, and the remaining cell-associated radioactivity was determined at the indicated times. Data are means $\pm$ S.E.M. of six experiments.

Fig. 4. Displacement of tracer insulin by unlabelled insulin in membranes from ATP-depleted (4 mM- NaN_3 treatment, ○) and control fat-cells (●).

After pre-incubation of cells obtained from a total of 12 animals in each group at separate days, membranes were prepared and stored in liquid N_2 . Binding was assayed in triplicate with the pooled membranes. The inset shows a Scatchard plot of the data.

Discussion

The present results indicate that ATP depletion reduced the affinity rather than the number of insulin

receptors in the adipocyte. This conclusion can be drawn from the displacement experiments in intact cells as well as in membranes. However, since insulin receptors represent a heterogeneous population of binding sites yielding curvilinear Scatchard plots, it is difficult to estimate alterations of number or affinity on the basis of binding curves alone. As the dissociation constant of the occupied receptor represents the quotient of dissociation and association rates, alterations of the receptor affinity may also emerge from kinetic data. These data indicated that ATP depletion increased the dissociation rate of the insulin-receptor complex, thereby increasing its dissociation constant. In accordance with the conclusion drawn from the binding curves, a decrease in affinity of the receptors results.

Three arguments support the conclusion that ATP depletion and reduction of receptor affinity are closely linked in a causal way, or follow the same causal event. First, insulin binding can be reduced by agents that directly or indirectly impair the energy-generating metabolism of fat-cells via different mechanisms: NaN_3 , theophylline (Joost & Steinfelder, 1983), dinitrophenol and sodium cyanide (Häring *et al.*, 1981). Secondly, the gradual decrease of ATP levels was paralleled by a reduction of insulin binding in an identical concentration range of NaN_3 . Thirdly, restoration of normal ATP levels fully reversed the effect of NaN_3 on insulin binding. The receptor-bound insulin is internalized by an energy-dependent process, and any effect produced by metabolic inhibitors might reflect a block of the so-called insulin processing. In support of this view it has previously been reported that the rapid translocation of receptor-bound insulin into intracellular compartments during the first minutes of incubation was blocked by metabolic inhibitors (Kono *et al.*, 1977). The effect of NaN_3 , as observed in the present study, however, required longer incubation times and was still visible in the plasma-membrane preparation of pretreated cells. Thus it is unlikely that the effects of NaN_3 are simply due to an inhibition of insulin processing, but rather they reflect an alteration of the receptor structure or of its environment in the cell membrane.

ATP-dependence of receptor affinity has previously been observed in the adrenoceptor (Baer & Porzig, 1980), and was attributed to a (supposed) decrease of the cellular GTP levels. GTP binds to a nucleotide regulatory subunit, which then activates adenylate cyclase, and may control the affinity of adrenoceptors (Rodbell, 1980). A similar regulation of insulin receptor affinity by nucleoside triphosphates might therefore be considered. At present, however, scant experimental support is available for this hypothesis. The effects of nucleoside triphosphates on insulin binding were small compared with those modulating the affinity of adrenoceptors, and

were produced by considerably larger concentrations (Cuatrecasas, 1971).

The ATP depletion of cells probably impairs phosphorylation reactions in the plasma membrane. It has recently been shown that the β -subunit of the insulin receptor is phosphorylated, and that insulin stimulates the phosphorylation in parallel to the occupation of the receptors (Kasuga *et al.*, 1982). It is tempting to speculate, therefore, that phosphorylation or dephosphorylation reactions regulate the receptor affinity, involving either the receptor or some closely related membrane protein.

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References

- Bachmann, E., Allman, D. W. & Green, D. E. (1966) *Arch. Biochem. Biophys.* **115**, 153–164
- Baer, M. & Porzig, H. (1980) *FEBS Lett.* **111**, 205–208
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Caro, J. F. & Amatruda, J. M. (1980) *Science* **210**, 1029–1031
- Corin, R. E. & Donner, D. B. (1982) *J. Biol. Chem.* **257**, 104–110
- Cuatrecasas, P. (1971) *J. Biol. Chem.* **246**, 7265–7274
- Draznin, B., Solomons, C. C., Emler, C. A., Schalch, D. S. & Sussman, K. E. (1980) *Diabetes* **29**, 221–226
- Fain, J. N. (1973) *Mol. Pharmacol.* **9**, 595–604
- Fehlmann, M., Carpentier, J.-L., LeCam, A., Thamm, P., Saunders, D., Brandenburg, D., Orci, L. & Freychet, P. (1982) *J. Cell Biol.* **93**, 82–87
- Filetti, S., Takai, N. A. & Rapoport, B. (1981) *Endocrinology* **108**, 2409–2411
- Gliemann, J., Østerlind, K., Vinten, J. & Gammeltoft, S. (1972) *Biochim. Biophys. Acta* **286**, 1–9
- Gordon, P., Carpentier, J. L., Freychet, P., LeCam, A. & Orci, L. (1978) *Science* **200**, 782–784
- Häring, H. U., Biermann, E. & Kemmler, W. (1981) *Am. J. Physiol.* **240**, E556–E565
- Joost, H. G. & Steinfelder, H. J. (1982) *Mol. Pharmacol.* **22**, 614–618
- Joost, H. G. & Steinfelder, H. J. (1983) *Mol. Cell. Biochem.* in the press
- Joost, H. G., Arend, W. & Holze, S. A. (1982) *Biochem. Pharmacol.* **31**, 1227–1231
- Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982) *Science* **215**, 185–187
- Kono, T., Robinson, F. W., Sarver, J. A., Vega, F. V. & Pointer, R. H. (1977) *J. Biol. Chem.* **252**, 2226–2233
- Newby, A. C., Luzio, J. P. & Hales, C. N. (1975) *Biochem. J.* **146**, 625–633
- Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380
- Rodbell, M. (1980) *Nature (London)* **284**, 17–22
- Steinfelder, H. J. & Joost, H. G. (1982) *Biochem. Biophys. Res. Commun.* **104**, 45–51
- Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513
- Yu, K. T. & Gould, M. K. (1977) *Biochem. Biophys. Res. Commun.* **77**, 203–210