Photoaffinity Labelling of the Insulin Receptor in Intact Rat Hepatocytes, Mouse Soleus Muscle, and Cultured Human Lymphocytes

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Summary. Using the photoreactive, biologically active insulin analogue, B2-(2 nitro, 4-azidophenylacetyl)des-Phe^{B1} insulin, which can be covalently bound to receptor molecules upon photolysis, the insulin receptor has been studied in three different types of cells or tissues: isolated rat hepatocytes, intact murine soleus muscle and cultured human lymphocytes. When compared with native insulin, this analogue displayed a slightly reduced binding affinity. Accordingly, the biological potency of the photoreactive analogue was decreased by approximately 30% compared with native insulin when tested for its ability to stimulate amino acid transport in hepatocytes, and deoxyglucose uptake in soleus muscles. It was as effective as insulin, however, at maximally stimulating concentrations

and therefore is a full insulin agonist. This photoprobe was used to specifically label the insulin receptor in the three tissues: after ultra-violet irradiation, sodium dodecyl sulphate-polyacrylamide gel analysis of extracts under reducing conditions revealed that most of the radioactivity was associated with a 130,000 dalton band. In isolated hepatocytes, two bands at 125,000 and 23,000 daltons were also specifically labelled. In three different cell types from three different animal species, the 130,000 dalton band appeared to be the major subunit of the insulin receptor.

Key words: Insulin, insulin receptors, mouse skeletal muscle, rat hepatocytes, human lymphocytes, photoaffinity labelling.

Binding of insulin to specific receptors located on the surface of target cells is the first step in the hormone's action. Recently, two methods have been developed which result in a non-reversible, covalent binding of insulin to its receptors. These methods involve either a chemical cross-linking of the hormone to its receptor [1, 2], or the use of photoreactive insulin analogues which upon photolysis can be covalently bound to receptor molecules [3–6]. Covalent binding of radiolabelled insulin to membrane components has allowed considerable progress in the knowledge of the subunit structure of the insulin receptor. Most studies, however, involving either the chemical cross-linking of insulin to its receptor [1, 2] or the use of photoreactive insulin [3–6] have been performed on membrane preparations, which precludes an exploration of the dynamics of the receptor in the living cell. The present study was thus designed not only to study the binding properties and biological activity of the photoreactive analogue B2-(2 nitro, 4-azidophenylacetyl)des-Phe^{B1} insulin, but more importantly to investigate the possibility of labelling the insulin receptor *in situ* in intact isolated rat hepatocytes, murine soleus muscle and cultured human lymphocytes.

Materials and Methods

Chemicals

The photoreactive insulin analogue, B2(2 nitro, 4-azidophenylacetyl)des-Phe^{B1} insulin, was prepared as previously described [6–8] and iodinated in the dark to a specific activity of 200–250 $\mu\text{Ci}/\mu\text{g}$ using a modification of the chloramine T method [referred to as the second modification in reference 9]. Porcine monocomponent insulin was generously supplied by the Novo Research Institute, Copenhagen, Denmark. Na^{125}I was purchased from the Commissariat à l'Energie Atomique, Saclay, France; α -amino-1(¹⁴C)isobutyric acid and 2-deoxy-D-1(¹⁴C)glucose were from the Radiochemical Centre, Amersham, UK. Bovine serum albumin, α -aminoisobutyric acid, gentamycin, bacitracin, phenylmethylsulphonyl fluoride (PMSF) and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes) were from Sigma, St. Louis, USA. RPMI 1640 medium (Roswall Park Memorial Institute Medium) was from Gibco Bio-Cult Ltd (Paisley, UK). All other reagents were of the best grade commercially available.

Isolated Hepatocytes, Liver Plasma Membranes, and Soleus Muscles

Hepatocytes were isolated from male Wistar rats (120–150 g) by collagenase digestion of the liver [10] and incubated in Krebs-Ringer bicarbonate buffer (pH 7.4), containing 10 mg/ml dialysed bovine se-

rum albumin (Fraction V), gentamycin (50 µg/ml), bacitracin (0.8 mg/ml) and PMSF (2 mmol/l) and gassed with O₂:CO₂ (95:5, v:v). The effect of the photoreactive insulin and native insulin on α -aminoisobutyric acid influx was investigated in isolated hepatocytes exposed to varying concentrations of peptides for 2 h (this time was required for the full expression of the hormonal effect [10]). Soleus muscles were isolated from 8-week-old male Swiss albino mice and incubated in Krebs Ringer bicarbonate buffer (pH 7.4), containing defatted bovine serum albumin (20 mg/ml) and pyruvate (2 mmol/l), and gassed. The uptake of 2-deoxyglucose in muscles was measured at 37 °C as previously described [11] in the absence or presence of varying concentrations of native or photoreactive insulin. All biological assays were conducted in the dark. Partially purified liver plasma membranes (step 11 in reference 12) were used.

Cultured IM-9 Lymphocytes

Human cultured lymphocytes of the IM-9 line were grown at 37 °C in RPMI 1640 medium containing 10% fetal calf serum. Glutamine (0.3 mg/ml) was added just before feeding. Cells were fed three times a week by dividing the culture 1:3 and adding fresh medium. Cells in late log phase or early stationary phase of growth were split 1:2 in fresh medium 24 h before use. The cells were removed from the growth medium by centrifugation at 600 g for 5 min at 24 °C and washed once by resuspension in the assay buffer. The washed cell pellet was then equilibrated in assay buffer at 15 °C. Approximately 2×10^6 cells/ml were incubated in assay buffer (50 mmol/l sodium Hepes; 120 mmol/l NaCl; 1.2 mmol/l magnesium sulphate; 1 mmol/l EDTA; 10 mmol/l glucose; 15 mmol/l sodium acetate; 10 mg/ml bovine serum albumin, and 2 mmol/l PMSF; pH 7.6).

Binding Studies

The binding of ¹²⁵I-insulin and ¹²⁵I-photoreactive insulin was measured in the dark, under steady state conditions, as previously described for isolated hepatocytes [13], isolated soleus muscles [11], cultured human lymphocytes [14], and liver plasma membranes [9] using 0.3 ng/ml (0.05 nmol/l) of native or photoreactive ¹²⁵I-insulin and varying concentrations of corresponding unlabelled insulin. For gel analysis, labelled insulin concentration was increased to 10 nmol/l.

Irradiation Procedure

All irradiations were conducted using a water-cooled high pressure mercury lamp (Philips HPK 125 W/L). The light was filtered through a black glass filter and the biological materials were irradiated for 3 min at a distance of 9 cm from the lamp, as described by Fehlmann et al. [8]. This procedure has been shown not to modify the morphology of the cells or their ability to transport actively aminoisobutyric acid [8]. The efficacy of the crosslinking procedure was 12–15% [8].

Polyacrylamide Gel Electrophoresis (PAGE)

At the end of irradiation, hepatocytes, soleus muscles, IM-9 lymphocytes and liver membranes were directly solubilized in 3% (w/v) boiling sodium dodecylsulphate (SDS) solution containing 10% glycerol (v/v), 2% β -mercaptoethanol (v/v) and 0.01% bromophenol blue (w/v). Lysates were further boiled for 4 min. Analysis of the samples by one-dimensional slab-gel, using a 5–15% linear gradient of acrylamide as a resolving gel and a 3% stacking gel, was performed according to Laemmli [15]. The gels were stained with Coomassie blue, fluorographed and vacuum dried; autoradiography was carried out by exposing the gels to Kodak X-OMAT-R film. The proteins used as molecular weight markers were: myosin heavy chain (200 K), β -galactosidase (116 K), phosphorylase b (94 K), bovine serum albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), soybean trypsin inhibitor (20 K), and lysozyme (14.4 K).

Results

Receptor-Binding Properties of Photoreactive Insulin

In the three biological systems tested (isolated rat hepatocytes, isolated mouse soleus muscles and human IM-9 lymphocytes), photoreactive insulin binding was 30–35% lower than native insulin binding at low peptide concentrations (Fig. 1). Scatchard analysis of the data (not shown) revealed that the photoreactive insulin analogue had a moderately reduced affinity (30–35% lower than that of native insulin) for the receptor, while it was capable of binding to the same number of total insulin receptors.

Biological Activity of Photoreactive Insulin

Insulin has previously been shown to stimulate amino acid transport in isolated rat hepatocytes [10, 16]. As shown in Figure 2a, maximal responses of α -aminoisobutyric acid transport were identical for the two insulins (approximately a twofold increase above basal). The concentration producing a half-maximal response was slightly higher for the photoreactive insulin (EC₅₀ approximately 1.7 nmol/l) than for native insulin (EC₅₀ approximately 1.1 nmol/l). In isolated soleus muscles, photoreactive insulin also maximally stimulated 2-deoxyglucose uptake (an index of the rate of glucose transport and phosphorylation) to the same extent as native insulin (Fig. 2b). As seen in hepatocytes, the EC₅₀ was higher with photoreactive insulin (approximately 0.5 nmol/l) than with native insulin (approximately 0.4 nmol/l).

Photoaffinity Labelling of Insulin Receptors

After the ¹²⁵I-labelled photoreactive insulin analogue had been allowed to bind in the dark, cells or muscles were irradiated, solubilized and extracts were analysed by SDS-PAGE under reducing conditions. As shown in Figure 3, the radioactivity was specifically located in a few bands: in hepatocytes (lane A), three bands were visible with molecular weights of 130,000, 125,000 and 23,000 daltons; in muscles (lane D) the radioactivity was associated predominantly with a 130,000-dalton band; in IM-9 lymphocytes (lane G), a 130,000 and a 98,000-dalton component were labelled. All those bands were attenuated or suppressed when unlabelled insulin at 10 nmol/l (lanes B and E) or 1 µmol/l (lanes C, F and H) was present during the association period. In order to elucidate whether the 123,000-dalton component, which was observed only with hepatocytes, was a characteristic of the liver insulin receptor or alternatively a degradation product generated during the isolation of the cells, liver plasma membranes were incubated with the photoreactive analogue (Fig. 4). When the binding was performed in the presence of the inhibitor of proteolysis PMSF, only one labelled band with a

Fig. 1. Binding of ^{125}I -photoreactive insulin ($\circ$ ---- $\circ$) and ^{125}I -insulin ($\bullet$ — $\bullet$) to rat hepatocytes, mouse soleus muscles, and cultured human lymphocytes. The ^{125}I -labelled peptide (0.05 nmol/l) was incubated with hepatocytes ($10^6/\text{ml}$) for 1 h at 20°C , with individual soleus muscles for 4 h at 20°C , and with IM-9 lymphocytes for 90 min at 15°C , in the absence or presence of varying concentrations of the corresponding unlabelled peptide. All experiments were carried out in the dark. Data are expressed as percentage of ^{125}I -peptide specifically bound. Results correspond to one representative experiment for hepatocytes and IM-9 lymphocytes. For soleus muscles, each point represent the mean $\pm$ SEM of six to eight individual muscles

Fig. 2a and b. Biological activity of photoreactive insulin ($\circ$ ---- $\circ$) and native insulin ($\bullet$ — $\bullet$) in hepatocytes and soleus muscles. Hepatocytes were incubated for 2 h at 37°C in the presence of varying concentrations of insulin; at the end of the incubation period α -aminoisobutyric acid (AIB) influx (0.1 mmol/l) was measured in 10 min assays as described in Materials and Methods. Soleus muscles were incubated for 60 min at 37°C with varying concentrations of peptide, and (^{14}C)-2-deoxyglucose uptake (0.5 mmol/l) was measured at the end of a 15-min incubation. Each point is the mean of triplicate determinations (hepatocytes), or the mean $\pm$ SEM of six to eight individual muscles

molecular weight of 130,000 was observed (lane A). However, when PMSF was omitted from the incubation mixture, a 125,000-dalton and a 23,000-dalton band were also present (lane C), which is similar to the situation found with isolated hepatocytes. All of those bands (i.e. 130,000; 125,000 and 23,000-dalton bands) were absent when membranes were incubated with an excess of unlabelled insulin (lanes B and D).

Discussion

A major breakthrough in the elucidation of the insulin receptor structure recently occurred with the development of photoaffinity probes and the use of bifunction-

al chemical agents to covalently crosslink radiolabelled insulin and receptor proteins [1–8, 17, 18]. In the present study, we have used a photoreactive analogue which is a full insulin agonist; this analogue displayed no decrease in maximal activity in the different systems tested (isolated hepatocytes and soleus muscle), while its binding affinity and its biological potency were slightly decreased when compared with native insulin. This analogue is thus a valid tool to investigate the structure and function of the insulin receptor.

The data reported in the present study show that the major insulin binding subunit labelled in situ in two intact cell types (isolated rat hepatocytes and cultured human lymphocytes) and in an intact isolated organ (the mouse soleus muscle) is a 130,000-dalton component.

Fig. 3. Photoaffinity labelling of insulin receptors in isolated hepatocytes, soleus muscles, and IM-9 lymphocytes. Samples were incubated in the dark with 10 nmol/l of ^{125}I -photoreactive insulin for 2 h at 15 °C (hepatocytes and lymphocytes) or 4 h at 20 °C (muscles) in the absence (–) or presence of 10 nmol/l (+) or 1 $\mu\text{mol/l}$ (++) of unlabelled insulin. The samples were then ultra-violet-irradiated and processed for SDS-PAGE as described in Materials and Methods. OR = origin

Fig. 4. Photoaffinity labelling of insulin receptor in liver plasma membranes. Purified liver plasma membranes (0.25 mg protein/ml) were incubated in the dark for 2 h at 15 °C with 7.5 nmol/l of ^{125}I -photoreactive insulin in the presence or absence of PMSF, and without (lanes A and C) or with (lanes B and D) 5 $\mu\text{mol/l}$ unlabelled insulin. The samples were then ultra-violet-irradiated, and processed for SDS-PAGE as described in Materials and Methods. OR = origin

This result agrees with that obtained after crosslinking of insulin and its receptor using a bifunctional reagent, and analysis of solubilized proteins by gel electrophoresis under reducing conditions [17, 18]. A minor 98,000 dalton band could also be detected in IM-9 lymphocytes. The existence of such a smaller subunit of the insulin receptor has been the subject of controversy in the past. This band was heavily labelled when biosynthetic labelling of the receptor was performed using ^{35}S -methionine [19, 20] or ^3H -monosaccharides [20, 21], followed by immunoprecipitation of the receptor with a specific anti-receptor antibody. Such a band was observed also in liver membranes after photoaffinity labelling [4], but not in the present study. The fact that this 98,000 band was labelled in studies using affinity labelling and thus involving the binding site of the receptor, suggests that this 98,000 subunit is one (or part) of the binding site(s) for insulin. It is presently unclear why this band was de-

tected only in IM-9 lymphocytes in our study. The present data do not exclude the possibility that the 98,000-dalton component represents a proteolytic fragment of the 130,000-dalton subunit. In isolated hepatocytes, in addition to the 130,000-dalton subunit, two bands (125,000 and 23,000 daltons respectively) were also specifically labelled. The fact that those bands were also present in liver membranes, but only when membranes were incubated in the absence of a protease inhibitor, suggests that these two bands in hepatocytes represent proteolytic fragments of the 130,000-dalton receptor subunit.

In conclusion, we have shown that: (1) the B2-photoreactive insulin derivative is a full insulin agonist with a slightly reduced affinity and biological potency; (2) this photoprobe can be used to specifically label the insulin receptor in intact cells in situ and even in an isolated organ; (3) in three different cell types from three dif-

ferent species (rat, mouse, and man) the specific labelling predominantly involves a 130,000-dalton band, which appears to be the major recognition moiety of the insulin receptor. Photoaffinity labelling of the insulin receptor in intact cells provides us with a powerful tool to investigate the fate of the insulin-receptor complex in intact cells.

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