Biochemical and Morphological Evidence That the Insulin Receptor Is Internalized with Insulin in Hepatocytes

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ABSTRACT There is morphological and biochemical evidence that insulin is internalized in hepatocytes. The present study was designed to investigate the fate of the insulin receptor itself, subsequently to the initial binding step of the hormone to the hepatocyte plasma membrane. The insulin receptor was labeled with a $^{125}$I-photoreactive insulin analogue (B2[2-nitro,4-azidophenylacetyl]des-Phe$^B$1-insulin). This photoprobe was covalently coupled to the receptor by UV irradiation of hepatocytes after an initial binding step of 2-4 h at 15°C. At this temperature, only limited (~20%) internalization of the ligand occurred. In a second step, hepatocytes were resuspended in insulin-free buffer and further incubated for 2-4 h at 37°C. After 2 h at 37°C, no significant radioactivity could be detected in non-UV-irradiated cells, whereas 12-15% of the radioactivity initially bound remained associated to UV-irradiated cells. Morphological analysis after electron microscopy revealed that ~70% of this radioactivity was internalized and preferentially associated with lysosomal structures. SDS PAGE analysis under reducing conditions revealed that most of the radioactivity was associated with a 130,000-dalton band, previously identified as the major subunit of the insulin receptor in a variety of tissues. Internalization of the labeled insulin-receptor complex at the end of the 37°C incubation was further demonstrated by its inaccessibility to trypsin. Conversely, at the end of the association step, the receptor (also characterized as a predominant 130,000-dalton species) was localized on the cell surface since it was cleaved by trypsin. We conclude that in hepatocytes the insulin receptor is internalized with insulin.

Evidence has accumulated that insulin, following its binding to specific receptors located on the surface of target cells, is progressively internalized and degraded in intracellular structures (1–4). Studies aimed at demonstrating insulin internalization mainly involved morphological techniques and the use of radiolabeled or fluorescently labeled insulin. Although the results have unequivocally shown that the hormone enters the cell, the fate of the receptor itself remains largely unknown. We therefore decided to examine this problem by taking advantage of a photoreactive insulin analogue to covalently label the insulin receptor in hepatocytes. The basic questions we wanted to address were: (a) is the receptor internalized with insulin and (b) is the receptor degraded during the internalization process?

The biochemical and morphological data presented in this paper indicate that the insulin receptor, covalently bound to a photoreactive insulin analogue, is internalized and preferentially associated with lysosomal structures as molecular species similar to those present on the cell surface.

MATERIALS AND METHODS

Isolation and Incubation of Hepatocytes

Hepatocytes were isolated from male Wistar rats (120–150 g) by collagenase dissociation of the liver as previously described (5). All experiments were carried out in a Krebs-Ringer bicarbonate (KRb) buffer, pH 7.4, containing 10 mg/ml bovine serum albumin (fraction V), gentamycin (50 μg/ml), bacitracin (0.8 mg/ml), and phenylmethylsulfonyl fluoride (PMSF, 2 mM), and gassed with a mixture of 5% CO$_2$/95% O$_2$. Hepatocytes were used immediately after isolation.
iodination of Insulin

The photoreactive insulin analogue, B[32-nitro-4-azidophenylacetyl] des-PheNH2-insulin, whose Npa group displays a strong absorption band at 330 nm (6), was prepared as previously described (6). Native insulin and the photoreactive insulin analogue were iodinated to a specific activity of 200-250 μCi/mg using a modification (referred to as "the second modification" in reference 7) of the chloramine-T method.

Incubation Procedures for Binding Experiments

Suspensions of isolated hepatocytes (1 x 10⁶ cells/ml) and 125I-labeled photoreactive insulin or 125I-labeled photoactive insulin were incubated for 2-4 h at 15°C, conditions that permitted steady-state binding. At the end of this association step, cells were collected by centrifugation, resuspended in the same volume of insulin-free buffer, and exposed or not to UV light. Hepatocytes were then diluted 20-fold in insulin-free buffer and incubated for 2-4 h at 37°C. These conditions have previously been shown to favor the release of intact and degraded 125I-insulin from hepatocytes (2, 8). Nonspecific binding was determined throughout in simultaneous experiments where unlabeled insulin (5 μM) was added to the incubation at the beginning of the association period. All data have been expressed as specific binding. The association step in all binding experiments was carried out in the dark, except where otherwise stated.

Irradiation Procedure

All irradiations were conducted, under standardized conditions, in a cold room (4°C) using a water-cooled high pressure mercury lamp (Philips HPK 125 W/1). The light was filtered through a "black glass" filter (U.V.W. 55; Hanau AG, FRG), which reduces the short UV emission intensities to very low levels (6). Suspensions (3 mm depth) of hepatocytes were irradiated at 330 nm for 3 min at a distance of 9 cm from the lamp. After UV irradiation under these conditions, hepatocytes were submitted to morphological and biological tests to assess the lack of cytotoxic effects of the procedure. Electron microscope examination of thin sections of control and UV-irradiated cells at the very end of the incubation procedure (i.e., after the 2-h incubation at 37°C that followed the 2-h incubation at 15°C) revealed a similar proportion (~25%) of damaged cells in both cell preparations. The capacity of UV-irradiated cells to actively transport α-aminoisobutyric acid and their hormone responsiveness were analogous to those of control cells (Table I).

Preparation of Cells for Electron Microscopy and Autoradiography

Cell pellets were fixed for 3-4 h at room temperature with 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The medium containing glutaraldehyde was then aspirated and replaced with 0.1 M phosphate buffer, pH 7.4. Processing of fixed samples for electron microscopy studies and the method used for quantitative electron microscopic autoradiography have been described in detail elsewhere (2). The only modifications concerned the morphometric determinations of the volume density of intracellular structures, which referred to the whole cytoplasmic volume excluding the nucleus (9).

Analysis of Cell-associated Radioactivity

Hepatocytes were rapidly sedimented by centrifugation and the cell pellets were immediately solubilized in boiling SDS solution (3% SDS, wt/vol) containing 10% (vol/vol) glycerol, 2% (vol/vol) β-mercaptoethanol, and 1% (wt/vol) bromophenol blue. Cell lysates were further boiled for 4 min. Analysis of the sample by one-dimensional slab-gel electrophoresis, using a 5-15% linear gradient of acrylamide as a resolving gel and a 3% stacking gel, was performed according to Laemmli (10). The gels were stained with Coomasie Blue, fluorographed, and vacuum-dried; autoradiography was carried out by exposing the gel to Kodak X-OMAT R film (11). The proteins used as molecular weight markers were myosin heavy chain (200,000), β-galactosidase (116,000), phosphorylase b (94,000), bovine serum albumin (67,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and lysozyme (14,400).

RESULTS

Binding Properties of the Photoreactive Insulin

Binding of the 125I-labeled photoreactive insulin analogue to isolated hepatocytes was measured in the presence of various concentrations of unlabeled analogue under steady-state con-

Association and Release of the Photoreactive Insulin

125I-labeled photoreactive insulin was first allowed to bind to isolated hepatocytes for 4 h at 15°C. The cells were then UV-irradiated or not, diluted in insulin-free buffer, and the
release of cell-associated radioactivity was followed as a function of time. Fig. 2 shows that 20% of the radioactivity bound at steady state was still associated with UV-irradiated hepatocytes after a 2-h release period. In control cells (not UV-irradiated), only 5–8% of the radioactivity initially bound was still associated with the cells after the same period. These results indicate that 12–15% of the radioactivity originally bound to hepatocytes at steady state is covalently linked to the cells after UV irradiation. Gel filtration analysis of cell extracts at the end of the 2-h association step at 15°C also revealed that after UV irradiation ~15% of the cell-associated radioactivity was in the form of a high-molecular-weight complex (data not shown).

Morphological Analysis of Cell-associated Radioactivity

At the end of the association step, the autoradiographic grains were predominantly located on the cell periphery (Fig. 3), and when their distribution was analyzed quantitatively most grains distributed around the plasma membrane (Table II). When the cells were then UV-irradiated and further incubated for 2 h at 37°C, 67% of the radioactivity that remained associated with the cells was internalized (Table II). Under these conditions, analysis of the intracellular distribution of autoradiographic grains according to the method previously described (2) indicated a preferential association of the labeled material with lysosomal structures (Fig. 4). In control cells (not UV-irradiated), the number of autoradiographic grains was indistinguishable from background.

Analysis of Cell-associated Radioactivity by SDS PAGE

Analysis of cell extracts by SDS PAGE under reducing conditions revealed that at the end of the association step and after photolysis, the radioactivity was located on three bands with molecular weights of 130,000, 125,000, and 23,000 (Fig. 5, lane A). When photolysis was omitted, virtually no labeling was observed (Fig. 5, lane B). The labeling of all three bands was absent when an excess of unlabeled insulin (5 μM) was present during the association period (not shown). Therefore, the covalent labeling of the 130,000-, 125,000-, and 23,000-dalton bands implicated the binding of insulin to its specific receptor. When photoactivation was followed by the 2-h incubation period at 37°C, SDS PAGE analysis revealed the same three labeled bands (Fig. 5, lane C), which were virtually undetectable when photoactivation had been omitted (Fig. 5, lane D).

Since morphological analysis indicated that ~70% of the covalent insulin-receptor complex was internalized after 2 h at 37°C, the similarity in the patterns of bands observed at the end of the association step at 15°C and after the subsequent incubation at 37°C strongly suggested that internalization was not accompanied by receptor degradation. Gel filtration analysis confirmed the formation of a high-molecular-weight complex after UV irradiation of hepatocytes subsequent to binding of the 125I photoprobe; after the 2-h incubation at 37°C, this complex was quantitatively recovered and represented >80% of the radioactivity remaining associated with the cells (data not shown).

Trypsin Sensitivity of Cell-associated Radioactivity

It is well-established that the insulin receptor is highly susceptible to degradation by trypsin (12, 13). We took advantage of this property to investigate the localization of the covalently linked cell-associated radioactivity, based on its accessibility to trypsin. The autoradiogram shown in Fig. 6 reveals that the 130,000- and 125,000-dalton bands were virtually absent when the cells were treated with trypsin at the end of association at 15°C, immediately after UV irradiation. Under this condition (Fig. 6, lane B), the radioactivity was located on two bands of 98,000 and 75,000 daltons that presumably represented degradation products of the native receptor subunits. When hepatocytes were instead exposed to trypsin at the end of the 2-h incubation at 37°C (which followed association at 15°C and UV irradiation), the pattern of labeling (Fig. 6, lane D) exhibited little alteration compared with that of non-trypsin-treated hepatocytes (Fig. 6, lane C). The loss of trypsin sensitivity of the cell-associated radioactive material is in agreement with the morphological observations and suggests that the covalent insulin-receptor complex is internalized.

| Time-course of association and release of 125I-photoreactive insulin in isolated hepatocytes: effect of UV irradiation. Hepatocytes (1 x 10⁸/ml) were incubated in the dark at 15°C with 0.3 ng/ml of 125I-photoreactive insulin. At the times indicated (association period) 0.25-ml triplicate samples of the cell suspension were centrifuged and the cell pellets were counted for 125I radioactivity. After a 4-h association period, half the cell suspension was UV-irradiated (open symbols) whereas the other half was kept in the dark (closed symbols). Hepatocytes were then collected by centrifugation, washed with cold buffer, resuspended in 100 ml of insulin-free warm KRB buffer, and incubated in the dark at 37°C. At the times indicated (release period), 5-ml samples of cell suspensions were centrifuged and the cell pellets were counted for 125I radioactivity.
| Table II | Percentage of Labeled Material Internalized after Incubation of Isolated Hepatocytes with 125I-Photoreactive Insulin |
|----------|---------------------------------------------------------------|
|          | Percent labeled material internalized*                       |
| 120 min 15°C | 19                                                            |
| 120 min 15°C + 3 min 15°C (UV irradiation) + 120 min 37°C | 67                                                            |
| 120 min 15°C + 120 min 37°C | ND                                                            |

* Based on previous determinations (2), 15% of the grains found inside the cell (>250 nm from the plasma membrane) was taken as a background level and the values presented in the table were corrected accordingly. ND, not detectable.
DISCUSSION

A major breakthrough in the elucidation of the insulin receptor structure recently occurred with the development of photoaffinity probes (12, 14–17) and the use of agents capable to covalently cross-link the hormone and the receptor (18). The latter technique, which allows the covalent binding of a large variety of ligands to cell surface receptors, may disturb the overall membrane architecture, and this would preclude the use of cross-linking agents to follow the fate of the receptor under conditions where hormone internalization has been demonstrated. To overcome this problem we have used a photo-reactive insulin analogue that retains ~70% of the receptor binding affinity and biological potency of native insulin in hepatocytes; under our experimental conditions, 12–15% of total cell-associated radioactivity was covalently bound to cellular components. SDS PAGE analysis under reducing conditions revealed that virtually all of the covalently bound radioactivity was linked to subunits of the insulin receptor. About 65% of the covalent labeling (i.e., 8–10% of total cell-bound radioactivity at the end of the association step) localized to a 130,000-dalton band, in agreement with previous reports on the covalent labeling of the insulin receptor in other cell types (17–19) and membranes (14–16, 20). We also observed two other bands of 125,000 and 23,000 daltons, which may represent degradation products of the 130,000-dalton species. Other
Hepatocytes (1 × 10⁸/ml) were incubated in the dark with 60 ng/ml of ¹²⁵I-photoreactive insulin for 2 h at 15°C in the presence of 2 mM PMSF. Half of the cell suspension was UV-irradiated and centrifuged; the cell pellets were then either directly solubilized in hot SDS (lane A), or resuspended in insulin-free buffer and incubated for 2 h at 37°C before analysis (lane C). The other half of the cell suspension was not UV-irradiated and was analyzed either before (lane B) or after (lane D) the 2-h incubation period at 37°C. An autoradiogram of the gel is presented. Molecular weight × 10³.

**FIGURE 4** Relationship of autoradiographic grains to intracellular organelles expressed as the ratio of the percentage of grains related to organelle over the volumetric density of organelles. **LIPID DR.**, lipid droplets; **SER**, smooth endoplasmic reticulum; **RER**, rough endoplasmic reticulum.

**FIGURE 5** Analysis of proteins from hepatocytes labeled with ¹²⁵I-photoreactive insulin by SDS PAGE under reducing conditions. Hepatocytes (1 × 10⁸/ml) were incubated in the dark with 60 ng/ml of ¹²⁵I-photoreactive insulin for 2 h at 15°C in the presence of 2 mM PMSF. Half of the cell suspension was UV-irradiated and centrifuged; the cell pellets were then either directly solubilized in hot SDS (lane A), or resuspended in insulin-free buffer and incubated for 2 h at 37°C before analysis (lane C). The other half of the cell suspension was not UV-irradiated and was analyzed either before (lane B) or after (lane D) the 2-h incubation period at 37°C. An autoradiogram of the gel is presented. Molecular weight × 10³. **OR**, origin.

Authors using cross-linking reagents (21) and biosynthetic labeling techniques (19) have reported the labeling of a 95,000-dalton subunit of the insulin receptor in cultured human lymphocytes. A 95,000-dalton band has been identified by photoaffinity labeling as a minor component of the insulin receptor in hepatoma cells (17). We also observed the labeling of a 95,000-dalton band in isolated hepatocytes, but only occasionally; by contrast, we found the 95,000-dalton band to be more consistently labeled in liver plasma membranes than in isolated hepatocytes (not shown). These differences cannot be explained by presently available data.

Analysis of the distribution of the radioactive grains by electron microscopy and autoradiography showed that the binding of the ¹²⁵I-photoreactive insulin to isolated hepatocytes at 15°C was accompanied by only ~20% internalization of the radioactivity. The morphological observation that internalization of covalent insulin-receptor complexes was minimal at the end of the association step (i.e., after 2 h at 15°C) was confirmed by trypsin treatment of the hepatocytes, which converted virtually all of the 130,000- and 125,000-dalton bands into smaller components. It has previously been shown that the internalization of ¹²⁵I-insulin is highly sensitive to temperature in isolated hepatocytes (2). Raising the temperature of the incubation medium containing hepatocytes previously exposed to the ¹²⁵I-photoreactive insulin and then UV-irradiated caused: a) a rapid release of the noncovalently bound ligand; and b) a redistribution of the radioactive grains, ~70% of which now became associated with intracellular structures. Under the latter conditions, most (>80%) of the cell-associated radioactivity eluted as a high molecular weight complex on gel filtration, and on SDS PAGE exhibited the same migration pattern (i.e., 130,000-, 125,000-, and 23,000-dalton bands) as that observed at the end of association. Furthermore, most of this cell-associated radioactivity was now virtually inaccessible to trypsin. Thus, both morphological and biochemical approaches indicate that the photolabeled insulin receptor is internalized in hepatocytes.

A rather intriguing observation was that the internalized receptor, although preferentially associated with lysosomal structures, was apparently not degraded since its pattern on SDS PAGE was similar to that of the receptor on the cell surface. The lack of degradation may have important biological implications, since internalization of insulin-receptor complexes is thought to be involved in the down-regulation of the receptor number on the cell surface (2). A lack of degradation of receptor proteins labeled with a photoreactive insulin derivative has also been recently reported in H4 hepatoma cells (17). One, however, should interpret this and our data with caution...
however, should be considered with caution because, in our experiments, only a relatively small fraction (12–15%) of the occupied receptors was covalently bound to the ligand and also because the mediator could have been generated during the association step, before photoactivation of the ligand.

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