Insulin Receptors in Isolated Human Adipocytes

CHARACTERIZATION BY PHOTOAFFINITY LABELING AND EVIDENCE FOR INTERNALIZATION AND CELLULAR PROCESSING

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ABSTRACT We photolabeled and characterized insulin receptors in isolated adipocytes from normal human subjects and then studied the cellular fate of the labeled insulin-receptor complexes at physiologic temperatures. The biologically active photosensitive insulin derivative, B2(2-nitro-4-azidophenylacetyl)des-Phe^{B1}-insulin (NAPA-DP-insulin) was used to photoaffinity label the insulin receptors, and the specifically labeled cellular proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

At saturating concentrations, the binding of ^{125}I-NAPA-DP-insulin to the isolated adipocytes at 16°C was rapid (half-maximal in ~1 min and maximal in ~10 min) and ~25% of the specifically bound ligand was covalently linked to the cells by a 3-min exposure to long-wave (366 nm) ultraviolet light. Analysis of the photolabeled cellular proteins by PAGE in the absence of disulfide reductants revealed the specific labeling of a major protein band of M_r 330,000 and two less intense bands of M_r 295,000 and 260,000. Upon reduction of disulfide bonds with dithiothreitol, all three unreduced forms of the insulin receptor were converted into a major labeled M_r-125,000 band and a less intensely labeled M_r-90,000 band. The labeling of the M_r-125,000 receptor subunit was saturable and native porcine insulin effectively inhibited (half-maximal inhibition at 12 ng/ml) the photolabeling of this binding subunit by NAPA-DP insulin.

When intact adipocytes photolabeled at 16°C (a temperature that inhibits endocytosis) were immediately trypsinized, all of the labeled receptor bands were converted into small molecular weight tryptic fragments, indicating that at 16°C all of the labeled insulin-receptor complexes remained on the cell surface. However, when the photolabeled cells were further incubated at 37°C and then trypsinized, a proportion of the labeled receptors became trypsin insensitive, indicating that this fraction has been translocated to the cell interior and thus was inaccessible to the trypsin in the incubation medium. The intracellular translocation of the labeled receptors was observed within 2 min, became half-maximal by 10 min, and maximal by ~30 min of incubation at 37°C. Cellular processing of the internalized insulin-receptor complexes also occurred, since incubation at 37°C (but not 16°C) resulted in the generation of a M_r-115,000 component from the labeled receptors. Inclusion of chloroquine, a drug with lysosomotropic properties, in the incubation media caused a time-dependent increase (maximal increase of 50% above control by 2 h at 37°C) in the intracellular pool of labeled receptors. In contrast to these findings in human adipocytes, no appreciable internalization of insulin-receptor complexes and no chloroquine effect

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was observed in cultured human IM-9 lymphocytes during a 1-h incubation at 37°C.

We conclude that in isolated human adipocytes: (*a*) the subunit structure of insulin receptors is the same as that reported for several other tissues, (*b*) insulin-receptor complexes are rapidly internalized and processed at physiologic temperatures, and (*c*) the cellular processing of insulin-receptor complexes occurs at one or more chloroquine-sensitive intracellular site(s).

INTRODUCTION

One of insulin's well-recognized biologic actions is the ability to regulate inversely the number of its cell surface receptors. This process, termed "down regulation," was first reported by Gavin et al. (1), using IM-9 lymphocytes, and has subsequently been demonstrated extensively in several other cell types (2–5). Furthermore, many pathophysiologic conditions such as obesity (6–8), impaired glucose tolerance (9, 10), and noninsulin-dependent diabetes mellitus (10–13) are characterized by varying degrees of hyperinsulinemia, decreased numbers of cell surface insulin receptors, and insulin resistance. Amelioration of hyperinsulinemia has also been shown to result in return of the cell surface receptor concentration to normal (14), thus suggesting that it is the hyperinsulinemia that leads to the receptor loss. In vitro evidence for this has been presented whereby incubation of isolated rat adipocytes with insulin led to a dose-dependent loss of cell-surface insulin receptors and a concomitant development of decreased sensitivity to insulin action at the glucose transport level (15). Thus, the accumulating evidence indicates that insulin-induced receptor loss or down regulation may serve an important regulatory function for overall insulin action. However, the specific mechanisms by which the process takes place and the metabolic fate of the insulin receptor remain poorly understood. In rat adipocytes (16, 17) and hepatocytes (18, 19), recent studies indicate that insulin receptors are internalized, but information regarding the cellular fate of insulin receptors in human tissues is lacking.

Although the isolated human adipocyte system has been used extensively for studies of insulin binding and insulin action in several pathophysiologic states, including obesity (8, 20, 21), impaired glucose tolerance (10), and diabetes mellitus (10, 22, 23), the biochemical characteristics as well as the cellular fate of insulin receptors in this major human insulin target cell system remain to be determined. The main reason for this is the fact that the small amounts of adipose tissue that can be obtained from patients for experimental studies are usually insufficient for detailed experimental characterizations using routine biochemical procedures. The technique of photoaffinity labeling of insulin receptors (16, 18, 24–26) affords a highly sensitive and attractive approach by which the biochemical characteristics and the cellular fate of insulin receptors can be studied in intact cells.

In this study, we used a biologically active photosensitive insulin derivative (*a*) to photoaffinity label and characterize insulin receptors in freshly isolated, viable human adipocytes, and (*b*) to study the cellular processing of insulin-receptor complexes in these cells.

METHODS

Materials. Carrier-free $Na^{125}I$ was purchased from Amersham Corp. (Arlington Heights, IL), collagenase from Worthington Biochemical Corp. (Freehold, NJ), and bovine serum albumin (BSA, fraction V) from Armour Pharmaceutical Co. (Chicago, IL). Eagle's minimal essential medium and soybean trypsin inhibitor were from Gibco Laboratories (Grand Island, NY). Phenylmethylsulfonylfluoride (PMSF),[1] *N*-ethylmaleimide (NEM), chloroquine (CQ), and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Porcine monocomponent insulin was generously supplied by Dr. Ronald E. Chance of Eli Lilly and Co. (Indianapolis, IN). Reagents for electrophoresis and marker proteins for molecular weight calibration were obtained from Bio-Rad Laboratories (Richmond, CA).

Photoreactive insulin derivative. The preparation and characterization of the photosensitive insulin analogue used in this study, B2(2-nitro-4-azidophenylacetyl)des-Phe^{B1}-insulin (NAPA-DP-insulin), have been described previously (27, 28). This derivative is biologically active and is a full insulin agonist at saturating concentrations (18, 29).

Iodination of NAPA-DP-insulin. NAPA-DP-insulin was iodinated in a dark room to a specific activity of 100–200 cpm/pg by using the water-insoluble oxidizing agent, 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (IODO-GEN; Pierce Chemical Co., Rockford, IL) essentially as we have described previously (16). The IODO-GEN was coated as a thin film (40 μg in 20 μl of chloroform) to the bottom of 12 × 75-mm glass tubes. 10 μg of NAPA-DP-insulin in 0.01 M HCl was reacted for 3 min with 3 mCi of $Na^{125}I$ in 200 μl of 0.1 M phosphate buffer (pH 7.5) in the IODO-GEN tube. The reaction mixture was then transferred to a clean test tube, and 50 μl of 10% BSA was added. This mixture was dialyzed against 0.1 M phosphate buffer (pH 7.5) containing 0.2% BSA to remove free ^{125}I, and the iodinated product was stored in the dark at 4°C and used within 1 to 2 wk.

Preparation of isolated human adipocytes and measurement of binding to intact cells. Adipose tissue specimens were obtained from 13 normal subjects (7 males, 6 females) by open biopsy from the lower abdominal wall as described previously (8, 21). The study subjects all had normal oral glucose tolerance and ranged in age from 23 to 73 yr (mean age, 37.9 yr). All biopsy procedures were performed in the

[1] *Abbreviations used in this paper:* CQ, chloroquine; IODO-GEN, 1,3,4,6-tetrachloro-3,6-diphenylglycoluril; NAPA-DP-insulin, B2(2-nitro-4-azidophenylacetyl)des-Phe^{B1}-insulin; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonylfluoride.

Clinical Research Center at the University of Colorado Health Sciences Center and informed consent was obtained in all instances.

Isolated adipocytes were prepared from the adipose tissue biopsy specimen by the collagenase digestion method of Rodbell (30) as described previously (15). Cell counts were performed according to a modification of method III of Hirsch and Gallian (31), in which the cells were fixed in 2% osmium tetroxide in 0.05 M collidine buffer (made isotonic with saline) for 24 h at 37°C and then taken up in a known volume of isotonic saline for counting. Counting was performed with a model ZB Coulter Counter with a 400-μm aperture (Coulter Electronics Inc., Hialeah, FL). Measurement of ^{125}I-labeled NAPA-DP-insulin (^{125}I-NAPA-DP-insulin) binding to intact cells was carried out as previously described (15).

Binding and photolysis of ^{125}I-NAPA-DP-insulin. Suspensions of isolated human adipocytes (~2.5 × 10^5 cells/ml) were incubated with various concentrations of ^{125}I-NAPA-DP-insulin in minimal essential medium containing 10 mM Hepes (pH 7.5) and 1% BSA. The incubations were carried out at 16°C in the presence or absence of 0.2 mM CQ in aluminum foil-covered plastic scintillation vials. Native porcine insulin, 20 μg/ml, was included where appropriate to determine nonspecific binding. After the desired incubation periods, the cells were transferred to 60 × 15-mm plastic petri dishes and photolysis was carried out for 3 min by exposure to a long-wave (366 nm) ultraviolet (UV) lamp (Blak-Ray; Ultra-Violet Products, Inc., San Gabriel, CA) placed 10 cm from the cell samples. The cells were then transferred to polypropylene tubes, washed three times, and solubilized with 1% sodium dodecyl sulfate (SDS) containing 1 mM NEM and 2 mM PMSF. The protocols for the experiments designed to study internalization and cellular processing of the labeled insulin-receptor complexes are described in the appropriate figure legends.

Polyacrylamide gel electrophoresis and autoradiography. The solubilized proteins from the photoaffinity labeled cells were analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (32). The samples were boiled for 2 min in Laemmli's sample buffer in the presence or absence of 25 mM dithiothreitol before application to the gels. The gels were stained with Coomassie Brilliant Blue, destained, dried, and autoradiographed at −70°C with Kodak X-Omat AR film and Cronex Lightning Plus (DuPont Instruments, Wilmington, DE) intensifying screen. The proteins used for standard molecular weight (M_r) calibration were: myosin (200,000), β-galactosidase (116,000), phosphorylase B (94,000), BSA (68,000), and ovalbumin (43,000).

RESULTS

Characteristics of binding and dissociation of NAPA-DP-insulin. Initial characterization of the time course of ^{125}I-NAPA-DP-insulin binding to isolated human adipocytes showed that binding was rapid at 16°C, becoming half-maximal within 1 min and maximal in ~10 min of incubation when saturating concentrations (60–70 ng/ml) of the photoactive insulin derivative were used (data not shown). Once a maximal (equilibrium) binding was reached at 16°C, it remained constant for up to 130 min of incubation.

Fig. 1 shows the effect of 3 min of UV light exposure on the dissociation (at 37°C) of bound ^{125}I-NAPA-DP-insulin from the adipocytes. After steady state binding at 16°C, dissociation of the bound ^{125}I-NAPA-DP-insulin at 37°C was rapid from the nonirradiated adipocytes and essentially 100% of the initially bound ligand was dissociated by 120 min of incubation. in contrast, 3 min UV exposure of the adipocytes after steady state binding at 16°C resulted in decreased dissociation of the bound ^{125}I-NAPA-DP insulin at 37°C. At the end of the 120-min dissociation period, ~25% of the specifically bound ligand still remained associated with the UV-exposed adipocytes. This represents the fraction of the bound ^{125}I-NAPA-DP-insulin that became covalently linked to the insulin receptors as a result of the photolytic reaction.

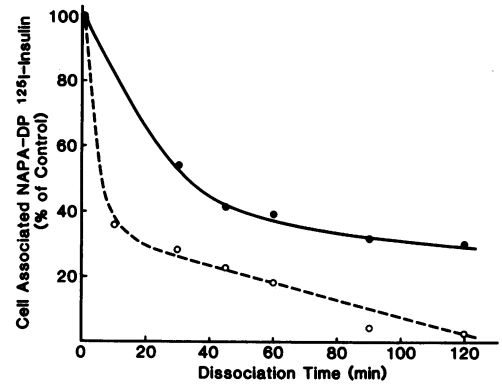

FIGURE 1 Effect of photolysis on dissociation of bound photoreactive insulin. Isolated adipocytes (2.7 × 10^5 cells/ml) were incubated with ^{125}I-NAPA-DP-insulin (60 ng/ml, 16°C, 60 min) in the presence or absence of excess unlabeled native insulin in a final volume of 3.3 ml. The samples were then quickly washed at 16°C and half of them were irradiated with UV light for 3 min. Both the irradiated (●) and nonirradiated (○) cells were then suspended in 4 ml of fresh buffer and further incubated at 37°C in the dark. At the indicated times, aliquots of the cell samples were removed and cell-associated ^{125}I-NAPA-DP-insulin was determined and corrected for nonspecific binding.

Identification of insulin receptors photolabeled in situ on intact adipocytes. Isolated human adipocytes, photolabeled at 16°C with ^{125}I-NAPA-DP-insulin, were solubilized and the labeled proteins analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Fig. 2 *A* shows the specific labeling of three unreduced forms of the insulin receptor with apparent M_r of 330,000, 295,000, and 260,000, when electrophoresis was performed in the absence of disulfide reductants. In this experiment, the photolabeled cells were solubilized with a 1% solution of the denaturing ionic detergent, SDS, containing 1 mM NEM and 2 mM PMSF to inhibit in vitro proteolysis. Under such conditions, the M_r-330,000 species appeared as the major

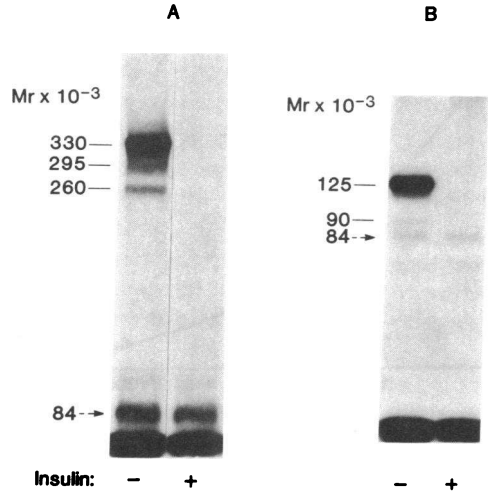

FIGURE 2 In situ photoaffinity labeling of insulin receptors on intact human adipocytes. Isolated adipocytes were incubated in the dark (16°C, 30 min) with 80 ng/ml ^{125}I-NAPA-DP-insulin in the absence (−) or presence (+) of excess unlabeled native insulin. The cells were then washed and subjected to photolysis as described in Methods. After further washing, the adipocytes were solubilized with 1% SDS containing 1 mM NEM and 2 mM PMSF. Aliquots of the solubilized samples were then either electrophoresed under nonreducing conditions on a 5% porous acrylamide gel with acrylamide/bisacrylamide ratio of 100:1 (*A*), or were reduced with dithiothreitol and electrophoresed on a 7.5% acrylamide gel (*B*). Autoradiograms of the dried gels are shown. The arrows indicate the nonspecifically labeled band.

labeled nonreduced form of the insulin receptor, while the M_r-295,000 and -260,000 species were labeled less intensely (Fig. 2 *A*). The M_r values of the unreduced forms of the insulin receptor reported in this study should be considered only as estimates, since they were obtained by extrapolation of the standard molecular weight calibration curve above M_r 200,000 and also because of the known anomalous mobility of glycoproteins in SDS-polyacrylamide gels.

When the solubilized proteins from the photolabeled adipocytes were treated with dithiothreitol and then electrophoresed, all of the nonreduced forms of the insulin receptor were converted into only two specifically labeled bands of M_r 125,000 and 90,000 (Fig. 2 *B*). In the reduced form of the receptor, almost all of the radioactive label is in the M_r-125,000 band, indicating that this subunit contains essentially all of the insulin binding sites of the receptor. The specific labeling of this binding subunit could be observed at ^{125}I-NAPA-DP-insulin concentrations as low as 5 ng/ml (8.3×10^{-10} M) and was saturable, reaching a maximum at 50–60 ng/ml of ^{125}I-NAPA-DP-insulin. In contrast, consistent photolabeling of the M_r 90,000 subunit was observed only at higher (≥30 ng/ml) NAPA-DP-insulin concentrations (data not shown). At ^{125}I-NAPA-DP-insulin concentrations as high as 90 ng/ml (15 nM), only the M_r-125,000 and -90,000 subunits of the insulin receptor were specifically labeled and no additional nonspecific bands were observed besides the single M_r-84,000 protein (indicated by arrows in Fig. 2 and in the other autoradiograms in this paper), thus demonstrating the high degree of specificity of the photoreactive insulin analogue for the insulin receptor. When equal amounts of solubilized samples from the photolabeled cells were electrophoresed under both reducing and nonreducing conditions, the results from the multiple experiments showed that the total amount of radioactivity in the reduced forms of the receptor was approximately half of that in the nonreduced forms (data not shown). This indicates that during disulfide reduction with dithiothreitol, the interchain disulfide bonds of ^{125}I-NAPA-DP-insulin are also reduced and the A-chain is then lost together with approximately half of the total ^{125}I-label in the iodinated insulin derivative; the B-chain remains covalently linked to the receptor.

Fig. 3 shows the competition by native porcine insulin for the labeling of insulin receptors by ^{125}I-NAPA-DP-insulin. Half-maximal inhibition of the labeling of the M_r-125,000 subunit occurred at native insulin concentrations of 12 ng/ml (2 nM). This finding, together with the results shown in Fig. 2, demonstrates that the NAPA-DP-insulin derivative specifically labels insulin receptors on isolated human adipocytes and that this labeling occurs at concentrations of the photoreactive insulin derivative that are well within the physiologic range of insulin concentrations.

Internalization and cellular processing of the labeled insulin-receptor complexes. The general experimental scheme that we have used to study the cellular fate of the photolabeled insulin receptors in human adipocytes is summarized in Fig. 4. Since endocytotic internalization is inhibited at 16°C (33), and since the insulin receptor is highly sensitive to tryptic degradation (34, 35), we used these facts to distinguish cell surface receptors from those that become translocated to the cell interior. Thus, at 16°C, essentially all of the labeled receptors should be on the cell surface and accessible to tryptic degradation. In contrast, further incubation of the photolabeled cells at 37°C would allow endocytosis and internalization to occur. Therefore, if insulin-receptor complexes are internalized in human adipocytes, then trypsinization of the photolabeled adipocytes at the end of the 37°C incubation should degrade only the receptors that remain on the cell surface while the internalized pool of receptors should be inaccessible to trypsin in the incubation medium.

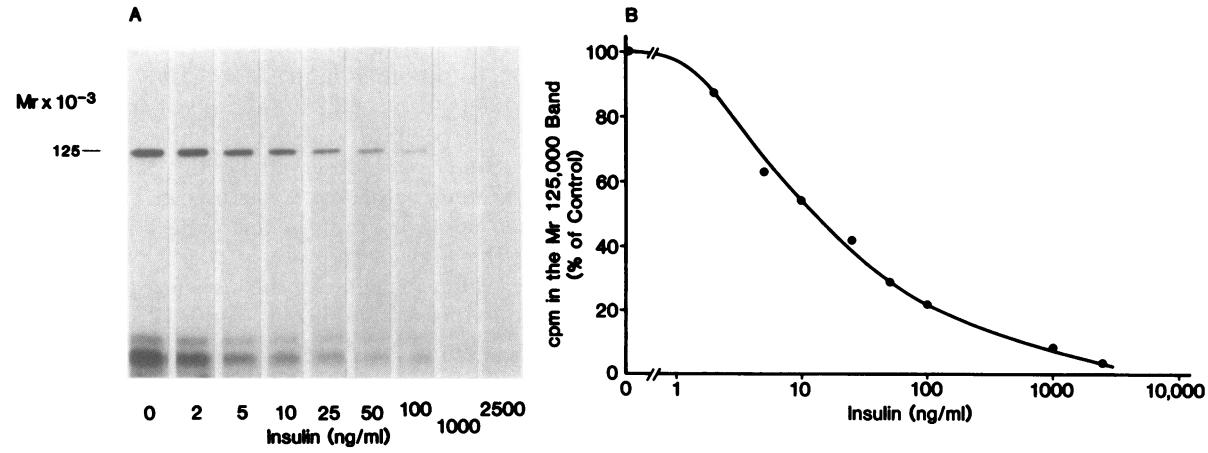

FIGURE 3 Competition by native porcine insulin for the photolabeling of insulin receptors. Adipocyte insulin receptors were photolabeled at 16°C with 20 ng/ml ^{125}I-NAPA-DP-insulin in the presence of the indicated concentrations of unlabeled porcine insulin. Shown are an autoradiogram of a 7.5% reducing gel (*A*), and a plot of the amount of radioactivity in the labeled M_r-125,000 band as a function of unlabeled insulin concentrations (*B*).

Using this experimental approach, we photolabeled insulin receptors on intact human adipocytes at 16°C and then studied the cellular distribution of the labeled receptors both at the end of the labeling process at 16°C and after a 1-h incubation of the labeled cells at 37°C. When adipocytes photolabeled at 16°C were trypsinized without further incubation at 37°C, all of the unreduced M_r-330,000, -295,000 and -260,000 insulin receptor species were completely converted into a major tryptic product of M_r 140,000 and a minor product of M_r 230,000 (Fig. 5), thus indicating that at 16°C, all of the labeled receptors were on the cell

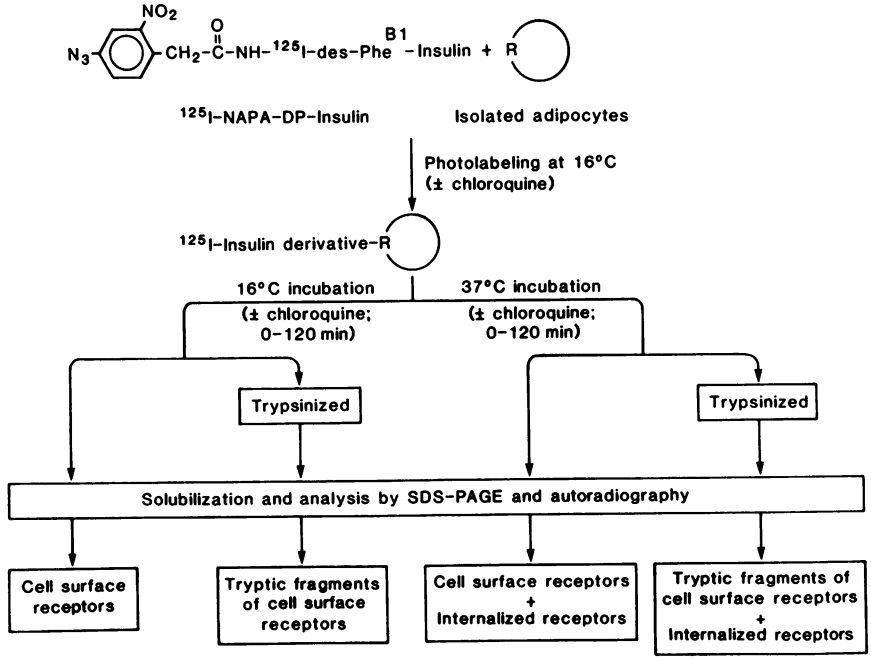

FIGURE 4 Experimental protocol used for studying the cellular processing of photolabeled insulin-receptor complexes.

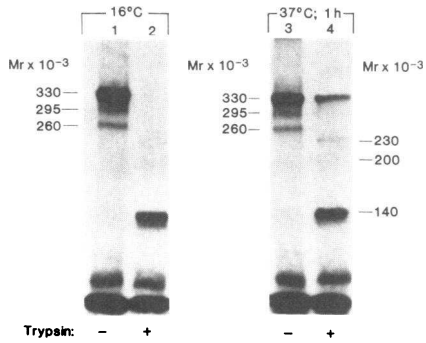

FIGURE 5 Generation of trypsin-insensitive (internalized) pool of photolabeled insulin-receptor complexes at 37°C. Photolabeling of adipocyte insulin receptors was carried out at 16°C as described in Methods and the labeled cells were then further processed according to the protocol described in Fig. 4. At the end of the 16°C photolabeling process, half of the labeled cells were immediately treated with trypsin as indicated, washed, and then solubilized. The rest of the labeled cells were incubated at 37°C for 1 h and were then treated with trypsin as indicated, washed, and solubilized. In each instance, trypsin (200 μg/ml) treatment was for 10 min at 37°C and the process was terminated by adding soybean trypsin inhibitor (400 μg/ml). Aliquots of the solubilized samples were analyzed on a 5% porous acrylamide gel under nonreducing conditions and autoradiogram of the dried gel is shown.

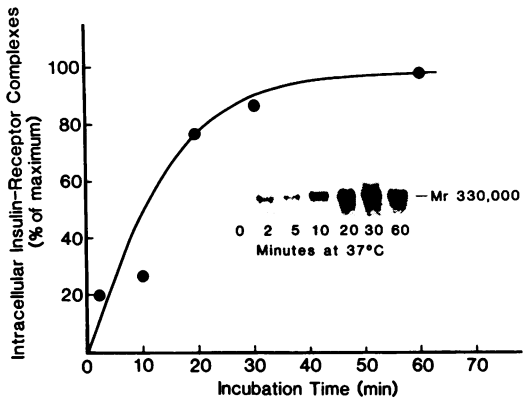

FIGURE 6 Time course of intracellular accumulation of insulin-receptor complexes at 37°C. Adipocytes photolabeled at 16°C were further incubated at 37°C and at the times shown, the cells were trypsinized to remove labeled receptors still remaining on the cell surface. The cells were then solubilized and subjected to electrophoresis (under nonreducing condition) and autoradiography. The areas of the dried gel containing the trypsin-insensitive (intracellular) M_r-330,000 receptor forms were cut out and the radioactivity was measured in a gamma counter. The results are expressed as percentages of the maximal amount of intracellular labeled insulin-receptor complexes and the data shown represent the mean from three separate experiments. The inset shows an autoradiogram depicting a time-dependent increase in the intensity of the M_r-330,000 trypsin-insensitive receptor band.

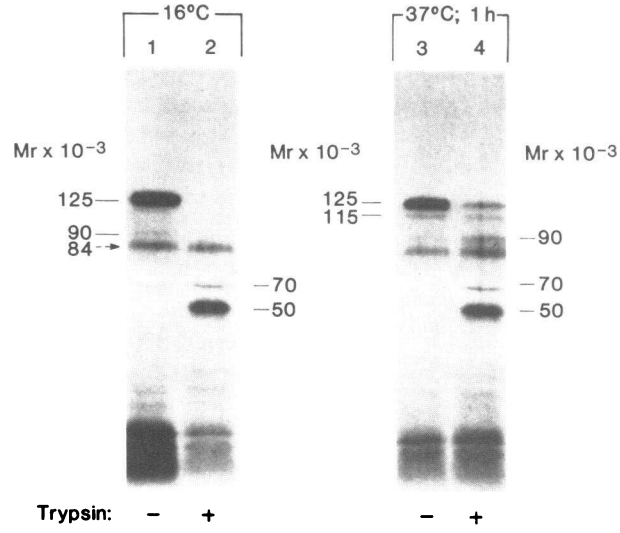

FIGURE 7 Autoradiogram showing the generation of a M_r-115,000 component. The experiment was carried out as detailed in Fig. 5, except that the solubilized samples were treated with dithiothreitol and then electrophoresed on a 7.5% acrylamide gel.

surface. In contrast, when the cells photolabeled at 16°C were further incubated at 37°C for 1 h and then trypsinized, a proportion of the M_r-330,000 receptor species became insensitive to tryptic degradation, indicating that this pool of receptors had been translocated to a trypsin-inaccessible (intracellular) compartment.

Fig. 6 shows the time course of the intracellular accumulation of labeled intact insulin receptors (M_r 330,000) at 37°C. It can be seen that trypsin-insensitive (intracellular) receptors could be observed within 2 min of incubation of the photolabeled cells at 37°C, indicating that internalization of insulin receptors in human adipocytes occurs very rapidly. The total intracellular pool of labeled receptors reached half-maximal amounts by 10 min and maximal amounts by ~30 min of incubation at 37°C; afterward, the amount of intracellular receptor pool remained relatively constant for up to 60 min of incubation at 37°C.

Fig. 7 shows the results obtained when the solubilized samples from an experiment identical to that described in Fig. 5 were first treated with dithiothreitol to reduce disulfide bonds and were then subjected to electrophoresis and autoradiography. As seen in lane 2 of this figure, all of the labeled M_r-125,000 and -90,000 insulin receptor subunits were completely degraded by trypsin treatment into smaller (predominantly M_r-50,000 and, to a lesser extent, M_r-70,000) proteolytic fragments, indicating that the receptor subunits were on the cell surface at 16°C. In contrast, when the photolabeled

adipocytes were further incubated for 1 h at 37°C, a proportion of the receptor subunits became insensitive to tryptic degradation (Fig. 7, lane 4), indicating that this pool had been translocated intracellularly. Additionally, a small amount of new labeled band of M_r 115,000 (which is trypsin insensitive) is also observed after incubation of the labeled cells at 37°C, indicating partial cellular processing of the internalized receptors. In Fig. 7 (lane 4), a tryptic product of M_r-90,000 (superimposed on any M_r-90,000 receptor subunit that might have been internalized) can also be seen when the labeled cells were trypsinized after 1 h incubation at 37°C, but not in the absence of trypsinization of these cells (lane 3) or when trypsinization was performed immediately after photolabeling at 16°C (lane 2). Thus, although this tryptic peptide happens to have the same apparent molecular weight as the M_r-90,000 subunit of the receptor, it is generated only upon trypsinization of cells incubated at 37°C and, therefore, must be different from the M_r-90,000 (β) subunit of the insulin receptor.

When the photolabeled adipocytes were trypsinized at various time points during their incubation at 37°C, and then solubilized and electrophoresed under disulfide reducing conditions, the time course of the generation of trypsin-insensitive (intracellular) labeled M_r-125,000 insulin receptor subunits (Fig. 8) was essentially the same as that of the unreduced M_r-330,000 receptor species (Fig. 6). Trypsin-insensitive M_r-125,000 subunits could be observed by 2 min and appeared to reach maximal amounts by 30–60 min of incubation at 37°C. During this same period, a time-dependent increase in the amount of the M_r-90,000 band can also be seen with a fall off occurring by 60 min. Since there was only minimal initial labeling of the M_r-90,000 (β) receptor subunit in this experiment (data not shown), the time-dependent increase in the M_r-90,000 band seen in Fig. 8 indicates an increase in the generation by trypsin of the M_r-90,000 proteolytic peptide (shown in Fig. 7, lane 4) with increasing time of incubation of the adipocytes at 37°C before trypsin exposure. The M_r-115,000 peptide is not well visualized in Fig. 8 because of the smaller amount of cells used per lane in this experiment as compared with those in Fig. 7. Fig. 8 also shows that after incubation of the labeled cells at 16°C for 30 and 60 min, no intracellular (trypsin-insensitive) M_r-125,000 receptor species and no M_r-90,000 tryptic products are seen.

The data shown in Figs. 5–8 indicated that once formed, insulin-receptor complexes remain on the adipocyte cell surface at 16°C, but that at 37°C, a proportion of them are internalized and processed. Since it has been shown previously that CQ, a drug with lysosomotropic effects (36, 37), can inhibit the cellular

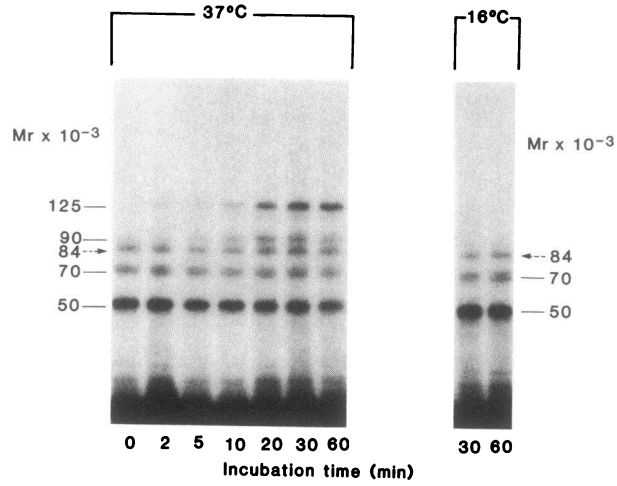

FIGURE 8 Time course of intracellular accumulation of M_r-125,000 insulin receptor subunits. Adipocytes photolabeled at 16°C were washed and further incubated at either 37 or 16°C. At the indicated times, the cells were trypsinized and solubilized. Electrophoresis of the solubilized samples was performed on a 5–15% linear acrylamide gradient gel under disulfide reducing conditions. Autoradiogram of the dried gel is shown.

degradation of ligands (38–40) and receptors (16, 17, 41), we next investigated the effects of this agent on the processing of insulin-receptor complexes by the isolated human adipocytes.

Effects of CQ on cellular processing of insulin-receptor complexes. CQ (0.2 mM) had no effect on the binding or photolabeling of insulin receptors at 16°C (Fig. 9 *A*, lanes 1 and 3). Furthermore, at 16°C, all of the labeled receptors remained on the cell surface in both the CQ-treated and untreated cells, since in each instance trypsin exposure degraded all of the labeled receptors (Fig. 9 *A*, lanes 2 and 4). In contrast, after 1-h incubation at 37°C, a proportion of the M_r-330,000 insulin receptor species became internalized in both the CQ-treated and untreated cells. However, in the presence of CQ, the intracellular pool of this receptor species was increased, as shown by the increased intensity of the M_r-330,000 band in the CQ-treated cells (compare lanes 6 and 8 in Fig. 9 *A*).

When the solubilized samples from the experiment described in Fig. 9 *A* were electrophoresed under disulfide reducing conditions, it was noted that after 1 h incubation at 37°C, the intracellular M_r-125,000 receptor pool was increased in the CQ-treated cells and furthermore, CQ also appeared to block the generation of the M_r-115,000 peptide (data not shown).

To assess the dynamics of insulin-receptor complexes in another human cell type, experiments identical to those described in Fig. 9 *A* were performed with cul-

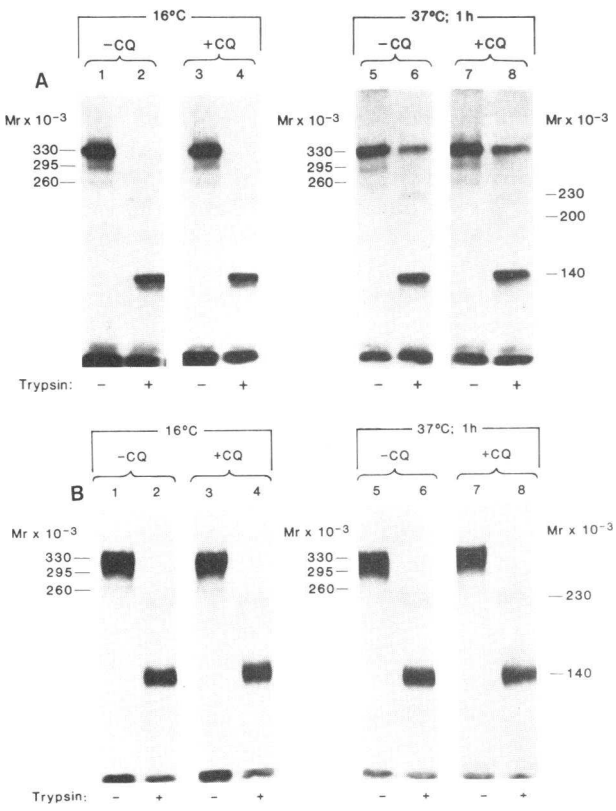

FIGURE 9 (A) Effect of CQ on cellular processing of insulin-receptor complexes in human adipocytes. Isolated adipocytes were photolabeled at 16°C in the absence (−*CQ*) or presence (+*CQ*) of 0.2 mM CQ as described in Methods. At the end of photolabeling at 16°C, half of the labeled cells were treated with trypsin as indicated and then solubilized. The rest of the labeled cells were incubated for 1 h at 37°C in the absence (−*CQ*) or presence (+*CQ*) of 0.2 mM CQ and then treated with trypsin as indicated and solubilized. Trypsin treatment was as described in Fig. 5. The solubilized samples were electrophoresed in a 5% porous acrylamide gel under nonreducing conditions and the autoradiogram of the dried gel is shown. (*B*) Lack of internalization of insulin-receptor complexes and of CQ effect in cultured human IM-9 lymphocytes. The experiment was performed exactly as described in *A*, except that cultured IM-9 lymphocytes were used.

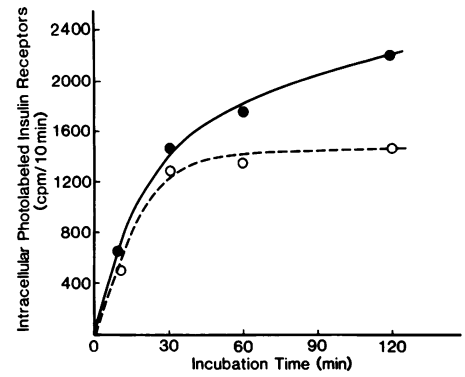

FIGURE 10 Time course of the effect of CQ in increasing the intracellular accumulation of insulin-receptor complexes at 37°C. Isolated human adipocytes photolabeled at 16°C were washed and further incubated at 37°C in the absence (○) or presence (●) of 0.2 mM CQ. At the indicated times, the cells were trypsinized and solubilized, and the samples were then analyzed by electrophoresis and autoradiography under nondisulfide-reducing conditions. The areas of the dried gel containing the trypsin-insensitive (intracellular) M_r-330,000 receptor species were cut out and the radioactivity measured in a gamma counter. The data shown are representative of three separate experiments.

tured human lymphocytes (IM-9), a widely studied cell line with high cell-surface insulin receptor concentrations, but in which there are no known glucoregulatory effects of insulin. Fig. 9 *B* shows that, unlike the finding in the adipocytes (Fig. 9 *A*), no trypsin-insensitive or intracellular insulin-receptor complexes are seen in the IM-9 lymphocytes when the photolabeled cells were incubated for 1 h at 37°C either in the absence (lane 6) or presence (lane 8) of 0.2 mM CQ.

Fig. 10 shows the time course of the effect of CQ on the intracellular accumulation of labeled receptors in the human adipocytes at 37°C. During the first 30 min of incubation, the effect of CQ to increase intracellular receptor accumulation was small. With a longer incubation period, however, the CQ effect became more pronounced and increasingly more labeled receptors were accumulated intracellularly between 60 and 120 min of incubation in the CQ-treated cells. By 120 min of incubation CQ caused an average of ~50% increase in the amount of labeled intracellular receptors.

DISCUSSION

Although it has recently been shown that insulin receptors are internalized in rat adipocytes (16, 17) and hepatocytes (18, 19), information regarding the metabolic fate of insulin receptors in human adipocytes has been lacking. The information derived from the animal cells, although very useful, cannot be directly extrapolated to human cell systems, since it is known that receptor regulation can occur by different mechanisms in different cell types and differences can also occur in the same cell types from different species. For example, whereas insulin receptors are internalized in rat adipocytes (16, 17) and hepatocytes (18, 19), they are shed into the incubation media by IM-9 lymphocytes (26). Lack of cellular insulin receptor processing has also been reported in H4 hepatoma cells, although biologic effects of insulin can be demonstrated in these

cells (42). Furthermore, in the β-adrenergic receptor system, it has been shown that hormone-induced desensitization of adenylate cyclase is associated with decreased receptor number in the frog erythrocyte, whereas in the turkey erythrocyte desensitization occurs without change in receptor number (43, 44). Such findings indicate that differences in the mechanism of regulation of receptor number and hormone sensitivity can occur among different cell types (16–19, 26, 42), and between the same cell types from different species (43, 44).

In the present study, we used a photoaffinity labeling technique to characterize insulin receptors in isolated human adipocytes and to follow the cellular fate of the labeled receptors at physiologic temperatures. The results show that (*a*) the subunit structure of insulin receptors in human adipocytes is the same as that previously reported for several other cell types; (*b*) insulin-receptor complexes remain on the cell surface at 16°C, but at 37°C these complexes are rapidly (within 2 min) internalized and their intracellular accumulation reaches a steady state within 30 min; and (*c*) the internalized insulin-receptor complexes are partially processed at CQ-sensitive intracellular sites, although the magnitude of the CQ effect appears to be less than that observed in rat adipocytes (16, 17).

The NAPA-DP-insulin derivative used in our studies has been well characterized and retains almost all of the biological activity and receptor binding affinity of native porcine insulin (18, 27–29, 45). At saturating concentrations, the binding of this derivative to isolated human adipocytes at 16°C was very rapid. Upon photolysis with a long-wave (366 nm) UV light source, ~25% of the specifically bound ligand could be covalently linked to the cells (Fig. 1) and this process did not affect cell viability, as assessed by trypan blue exclusion. Analysis of the covalently bound radioactivity by gel electrophoresis and autoradiography revealed the specific labeling of insulin receptor proteins (Fig. 2) and only a single cell surface protein of M_r-84,000 was nonspecifically labeled in most of the experiments, thus indicating the high degree of specificity of NAPA-DP-insulin for the insulin receptor. Furthermore, physiologic concentration of native porcine insulin effectively inhibited the photolabeling of the receptors (Fig. 3), indicating that both native insulin and NAPA-DP-insulin bind to the same ligand recognition sites of the receptor.

In the nonreduced state, the labeled insulin receptor species appeared primarily in the M_r-330,000 form, and to a lesser extent in the M_r-295,000 and -260,000 forms (Figs. 2, 5, and 9 *A*). The appearance of similar heterogeneous forms of unreduced insulin receptors has also been reported previously (46). We have also shown (16) that when the nondenaturing detergent Triton X-100 was used for solubilization of photolabeled rat adipocytes, the nonreduced forms of the insulin receptor appeared primarily in the M_r-260,000 and, to a lesser extent, in the M_r-330,000 and -295,000 forms, thus indicating that conversion of the M_r-330,000 and -295,000 species into the M_r-260,000 form was occurring in vitro, possibly owing to the action of proteases co-solubilized with the labeled receptors. In the present study, although the use of 1% SDS (which, unlike Triton X-100, should denature proteases) partially reverses this conversion and yields the M_r-330,000 species as the major labeled form of the unreduced receptor, complete inhibition of the generation of the M_r-295,000 and -260,000 species was not achieved, despite inclusion of PMSF and NEM in the solubilizing solution (Figs. 2, 5, and 9 *A*). Thus, despite the likelihood that at least a portion of the heterogeneous forms of the unreduced insulin receptors are generated in vitro because of proteolysis during sample preparation (16, 46), it is also possible that some of these heterogeneous receptor species are generated on the cell surface, possibly as a result of the initial binding of insulin to the receptor. In this regard, it should be noted that a recent report has apppeared indicating that insulin affects the structural organization and binding characteristics of its own receptor in H35 rat hepatoma cells (47). Thus, in future studies it will be important to determine the precise proportions of the oligomeric unreduced insulin receptor forms that may be generated in other insulin target cells under different physiologic conditions. Upon complete reduction of disulfide bonds, all of the three unreduced forms of the insulin receptor were converted into only two subunits of M_r-125,000 and -90,000 (Fig. 2). Essentially all of the radioactivity was contained in the M_r-125,000 band, indicating that this subunit represents the major insulin binding component of the receptor (24–26, 46). Although the M_r-90,000 subunit does not participate in insulin binding to a major extent (Fig. 2), it is an integral component of the insulin receptor macromolecule, as shown by biosynthetic labeling (48) and receptor purification (49) studies. Furthermore, based on the results of a series of recent important studies (50–53), it is becoming clear that the phosphorylation of this (β) subunit is selectively stimulated by insulin and that this subunit itself has kinase activity (54). Thus, the emerging picture is that the M_r-125,000–135,000 (α) subunit serves as a ligand recognition component of the receptor, while the M_r-90,000–95,000 (β) subunit may be involved in the transduction or regulation of insulin action through specific insulin-induced phosphorylation of this subunit and other effector proteins.

In studying the cellular processing of the labeled

insulin receptors, we interpreted the data to signify that the trypsin-sensitive receptors represent those still on the cell surface, while the trypsin-insensitive fraction represents the internalized pool of receptors and their intracellular degradation products (Fig. 4). Differential sensitivity to trypsin has similarly been used previously to distinguish between cell surface and intracellular receptors for insulin (16–18) and epidermal growth factor (41). The results shown in Figs. 5–8 demonstrate that insulin-receptor complexes that remained on the adipocyte cell surface at 16°C were rapidly internalized and processed when the labeled cells were incubated at 37°C. Processing of the receptors is indicated by the generation of the M_r-115,000 component by 37°C incubation (Fig. 7). As shown in Figs. 5 and 9 *A*, trypsin-insensitive (internalized) forms of only the M_r-330,000 unreduced receptor species were observed. Since we could not detect trypsin-insensitive forms of the M_r-295,000 and -260,000 species, it is likely that the proportion of these two forms in the internalized pool may be too small to be detected by the technique we used. Alternatively, if these two forms of the receptor are generated on the cell surface, then it is possible either that they are not internalized or, if they are, that they are rapidly degraded.

The data in Figs. 6 and 8 show that at 37°C insulin-receptor complexes are translocated to the cell interior very rapidly (within 2 min) and that the intracellular accumulation of these complexes proceeds rapidly during the initial 30 min of incubation. Between 30 and 60 min of incubation, a steady state was reached and the intracellular pool of the insulin-receptor complexes remained constant. This suggests that after 30 min at 37°C either further receptor internalization becomes slowed or the rate of degradation of the internalized receptors comes into balance with the rate of internalization of receptors.

The parallel time-dependent increase in the amount of the tryptic M_r-90,000 peptide and the internalized pool of M_r-125,000 receptor species at 37°C (Fig. 8) suggest that the generation of the tryptic M_r-90,000 peptide may be related to the internalization of insulin-receptor complexes. This notion is further supported by the finding that at 16°C neither internalized receptors nor tryptic M_r-90,000 peptide are seen (Fig. 8). Furthermore, we have also recently found that in isolated rat adipocytes tryptic generation of the M_r 90,000 proteolytic peptide is observed at 37°C and that the time-dependent formation of this peptide is directly correlated with the internalization of insulin-receptor complexes (55). Thus, taken together, these results indicate that the internalization of insulin-receptor complexes occurs by similar mechanisms in both human and rat adipocytes.

Since CQ is known to have lysosomotropic effects (36, 37) and has also been shown to inhibit the intracellular degradation of internalized hormones and receptors (16, 17, 39–41), we investigated its effects on the processing of insulin-receptor complexes by human adipocytes. The results show that CQ caused an increase in the intracellular accumulation of the M_r-330,000 species (Fig. 9 *A;* compare lanes 6 and 8) and also an increase in the amount of the intracellular form of the M_r-125,000 subunit (data not shown). These findings suggest that the processing of insulin-receptor complexes occurs at CQ-sensitive intracellular sites. Although it is possible that CQ may affect other intracellular processes (56) besides inhibiting lysosomal function (36, 37), our results are consistent with the possibility that lysosomes are involved in the processing of internalized insulin-receptor complexes in human adipocytes. In Fig. 9 *B*, the absence of any demonstrable receptor internalization and the lack of CQ effect in the IM-9 lymphocytes, a human cell line in which the fate of insulin receptors has been shown to be different from that in insulin target cells (26), demonstrate the usefulness of the photoaffinity labeling approach in revealing differences in insulin receptor dynamics in insulin target and nontarget cells.

The observed minimal effect of CQ in increasing the intracellular accumulation of insulin-receptor complexes during the initial 30-min incubation at 37°C (Fig. 10) suggests that degradation of the internalized complexes, at least by CQ-sensitive mechanisms, is minimal during this period. The finding that a rapid increase in the intracellular accumulation of insulin-receptor complexes occurs during this same period (Fig. 6) is also consistent with this possibility, since a rapid increase in the internalized pool can occur only if there is a minimal degradation of this pool of receptors. The observation that the amount of intracellular insulin-receptor complexes reaches a plateau after 30 min at 37°C (Fig. 6) and the finding that CQ causes a more pronounced increase in the intracellular pool of these complexes during this same period (Fig. 10) indicate that significant degradation of the internalized insulin-receptor complexes begins after 30 min incubation at 37°C. This raises the possibility that insulin receptors are initially internalized into a subcellular compartment in which degradation of the receptors does not occur, and that after some time the internalized receptors are transported to a compartment in which degradation readily occurs.

The overall effect of CQ to inhibit the degradation of internalized insulin-receptor complexes in the human adipocytes is quantitatively less than its effects in rat adipocytes (16, 17). This difference in the magnitude of the CQ effect is unlikely to be due to the possibility

that internalization of insulin-receptor complexes occurs more slowly in human compared with rat adipocytes, since similar rapid rates of internalization are observed in both cell types (Figs. 6 and 8, and reference 55). Thus, it seems possible that the smaller CQ effect in human adipocytes may be due to a smaller proportion of overall receptor degradation via a CQ-sensitive pathway in these cells as compared with rat adipocytes. Furthermore, since the human adipocytes were obtained from subcutaneous adipose tissue, and the rat adipocytes from epididymal fat pads, differences in insulin-receptor metabolism may exist in the same cell types obtained from different regions of the body. In this regard, it is of interest to note that a recent report by Bolinder et al. (57) indicates that despite similar receptor numbers per cell, human subcutaneous and omental fat cells show marked differences in insulin receptor affinity and in their sensitivity to the antilipolytic effects of insulin. Additionally, variations in glucose metabolism and insulin response by fat cells isolated from three different adipose tissue depots of the rat have also been described (58).

It should be noted that in the present study we followed the fate of covalently linked insulin-receptor complexes. Since under normal physiologic conditions the interaction of insulin with its receptors is noncovalent, it can be doubted whether internalization and processing of covalently linked insulin-receptor complexes is different from that which occurs normally under physiologic conditions. In a previous study with rat adipocytes, however, we have demonstrated that intracellular insulin receptor can be identified by photoaffinity labeling after allowing insulin-induced receptor internalization to occur, and that CQ inhibited degradation of the internalized pool of receptors (59). Thus, the internalization of insulin receptors appears to occur by the same process, whether the fate of the receptor is followed without covalent modification (17, 59) or after covalent linking of insulin to the receptor (16) on the cell surface.

In summary, we used photoaffinity labeling techniques to characterize insulin-receptor complexes in isolated human adipocytes and demonstrated that at physiologic temperatures these complexes are rapidly internalized and subsequently processed at one or more CQ-sensitive intracellular sites. In future studies, it will be of interest to investigate the detailed pathways and subcellular locations of insulin receptor processing in human adipocytes and to determine whether abnormalities in these pathways exist in pathophysiologic states.

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