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Tyrosine phosphorylation of the insulin receptor is not required for receptor internalization: Studies in 2,4-dinitrophenol-treated cells

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ABSTRACT **The relation between insulin-stimulated autophosphorylation of the insulin receptor and internalization of the receptor was studied in Fao rat hepatoma cells. Treatment of Fao cells with 2,4-dinitrophenol for 45 min depleted cellular ATP by 80% and equally inhibited insulin-stimulated receptor autophosphorylation, as determined by immunoprecipitation of surface-iodinated or [^{32}P]phosphate-labeled cells with anti-phosphotyrosine antibody. In contrast, internalization of the insulin receptor and internalization and degradation of ^{125}I-labeled insulin by 2,4-dinitrophenol-treated cells were normal. These data show that autophosphorylation of the insulin receptor is not required for the receptor-mediated internalization of insulin in Fao cells and suggest that insulin receptor recycling is independent of autophosphorylation.**

Tyrosine autophosphorylation of the β-subunit of the insulin receptor and the phosphorylation of putative substrates are among the earliest cellular responses to insulin binding at the cell surface (1, 2). A growing number of studies using mutant receptor molecules and antibodies that inhibit receptor kinase activity suggest that the tyrosine kinase in the β-subunit plays a central role in the transmission of the insulin signal (3–6). Insulin also stimulates the rate of insulin receptor internalization in a variety of cell types (7–12). In Fao cells, internalization is half-maximal within 10 min, and most of the internalized receptors are phosphorylated on tyrosyl residues (13).

Mutation of Lys-1018* in the ATP binding domain of the insulin receptor abolishes receptor autophosphorylation and inhibits insulin-stimulated endocytosis of the insulin receptor and internalization and degradation of insulin in Chinese hamster ovary and Rat-1 cells (15–17). These results suggest that autophosphorylation or tyrosine kinase activity is required for receptor internalization. However, we have uncoupled these processes in Fao cells by reducing cellular ATP levels with 2,4-dinitrophenol (DNP). Our results show that receptor autophosphorylation and cellular ATP levels decreased in parallel during incubations with DNP, whereas receptor internalization and insulin uptake and degradation were unaffected by DNP. Thus, insulin-stimulated internalization of the normal insulin receptor occurred in the absence of tyrosyl autophosphorylation.

MATERIALS AND METHODS

Materials. DNP, lactoperoxidase, ATP, phenylmethylsulfonyl fluoride, aprotinin, soybean trypsin inhibitor, and glucose oxidase were purchased from Sigma. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was purchased from Worthington. [^{125}I]Iodine (350–500 Ci/ml; 1 Ci = 37 GBq) and ^{125}I-labeled insulin (^{125}I-insulin) (2000 Ci/mmol) were purchased from Amersham, and [^{32}P]orthophosphate was from New England Nuclear. RPMI 1640 medium was purchased from GIBCO. Insulin-free bovine serum albumin (BSA) was purchased from Arnell (New York). Porcine insulin was from Elanco (Indianapolis). Reagents for SDS/PAGE and protein determination were purchased from Bio-Rad and Pierce. Intensities of autoradiographic bands were determined with a Hoeffer GS 300 densitometer. ATP levels were measured by using the luciferin/luciferase assay (Sigma) as described by Larkin *et al.* (18).

Cell Culture. Fao cells are derived from the Reuber H35 rat hepatoma and have a high concentration of insulin receptors and many insulin-stimulated responses (19). The cells were maintained as previously described and used on the first day of confluence after an overnight incubation in serum-free RPMI 1640 medium (20). Cell viability after DNP treatment was assessed by exclusion of trypan blue and by the recovery of cellular ATP levels after withdrawal of DNP for 1 hr.

[^{32}P]Phosphate Labeling and Immunopurification of the Insulin Receptor from Fao Cells. Fao cells were labeled for 2 hr at 37°C in phosphate-free RPMI 1640 medium containing 0.5 mCi of [^{32}P]orthophosphate per ml as described (20). The radioactive medium was replaced with phosphate-free RPMI 1640 medium containing the indicated concentration of DNP. After various incubation times the cells were stimulated with 100 nM insulin for 3 min and frozen with liquid nitrogen. The cells were thawed and solubilized with "stopping solution" (50 mM Hepes/1% Triton X-100/10 mM Na_4PO_7/100 mM NaF/2 mM Na_3VO_4/2 mM phenylmethylsulfonyl fluoride/0.1 mg of aprotinin per ml). Insoluble material was removed by centrifugation, and the insulin receptor was immunopurified from the supernatant with anti-insulin receptor antibody (αIR) or anti-phosphotyrosine antibody (αPY) as described (21). Precipitated proteins were separated by SDS/PAGE on 7.5% resolving polyacrylamide gels under reducing conditions, the gels were fixed and dried, and the labeled proteins were detected by autoradiography (21). The autoradiograms were quantified by scanning densitometry as described (13).

Internalization and Degradation of ^{125}I-Insulin by Fao Cells. Fao cells were incubated at 37°C in "binding buffer" (RPMI 1640 medium containing 0.1% BSA/25 mM Hepes, pH 7.4) in the absence or presence of 2 mM DNP for 45 min. Fresh medium containing 200,000 cpm of ^{125}I-insulin per ml in the absence or presence of DNP was added to the cells and incubations were continued at 37°C. At the indicated times the medium was removed and saved for determination of insulin degradation by precipitation in 10% trichloroacetic acid at 4°C. The cells were twice washed for 3 min with either neutral phosphate-buffered saline (PBS) (pH 7.4) containing 0.1% BSA to remove free insulin or acidic PBS (pH 3) containing 0.1% BSA to remove free and surface-bound insulin. All of the cells were washed an additional time in

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Abbreviations: DNP, 2,4,-dinitrophenol; BSA, bovine serum albumin; αIR, anti-insulin receptor antibody; αPY, anti-phosphotyrosine antibody.

*The numbering sequence used in this paper was described by Ullrich *et al.* (14) and is based on the position of the amino acids in the precursor of the human insulin receptor.

neutral PBS and then solubilized in 0.5 M NaOH/0.1% SDS. The acid washing procedure was >95% effective in the removal of surface-bound ^{125}I-insulin (data not shown). Therefore, radioactivity present in acid-washed cells represents internalized insulin, whereas radioactivity present in neutral-washed cells represents total cell-associated insulin.

Insulin-Stimulated Loss of Surface Binding. Confluent and serum-starved Fao cells were incubated with binding buffer in the absence or presence of 2 mM DNP at 37°C for 45 min. Fresh binding buffer containing 100 nM insulin in the absence or presence of DNP was added to the cells at 37°C. At the indicated time intervals, the medium was removed and the cells were chilled with ice-cold PBS. Surface-bound insulin was removed by washing the cells twice with acidic PBS (pH 3) and once with neutral PBS as described above. The specific binding of 0.01 nM ^{125}I-insulin during 15 hr at 4°C was then determined as described (20). To demonstrate the efficacy of the washes in removing surface-bound insulin, Fao cells were incubated in 100 nM insulin at 15°C for 3 hr. This was followed by acid washing and a determination of specific insulin binding at 4°C; surface binding after the acid washes was >98% of basal.

Internalization and Tyrosyl Phosphorylation of Insulin Receptors in Surface-Iodinated Fao Cells. Confluent serum-starved Fao cells in 100-mm dishes were washed in ice-cold PBS. Cold PBS (3 ml) containing 10 mM glucose and 0.4 unit of lactoperoxidase per ml was added to the dishes and surface-labeling was initiated by the addition of PBS (30 μl) containing 1.25 mCi of ^{125}I-iodine and 100 units of glucose oxidase per ml. The cells were incubated for 30 min, at 4°C on a rocking platform, and the labeling was terminated by washing the cells five times at 4°C with PBS. Iodinated Fao cells were incubated at 37°C with binding buffer in the absence or presence of 2 mM DNP for 45 min. The cells were stimulated with 100 nM insulin for varying times, rapidly chilled, and incubated in the absence or presence of 0.5 mg of trypsin per ml (1 ml in PBS containing 2 mM Na_3VO_4) for 30 min at 4°C. After two washes with PBS containing 2.5 mg of soybean trypsin inhibitor per ml, the cells were solubilized and proteins were immunoprecipitated with either αPY or αIR and separated by SDS/PAGE as described above. Internalization was quantified by scanning densitometry of trypsin-resistant α-subunit bands as described (13).

RESULTS

Inhibition of Insulin Receptor Autophosphorylation by DNP. Tyrosyl autophosphorylation of the β-subunit of the insulin receptor was detected by immunoprecipitation of [^{32}P]phosphate-labeled proteins from Fao cells with αPY. Incubation of Fao cells with 2 mM DNP significantly reduced insulin-stimulated tyrosyl autophosphorylation of the β-subunit (Fig. 1*A*). The inhibition was half-maximal after 10 min of DNP treatment and >90% after 45 min (Fig. 1*B*). To determine whether DNP inhibited insulin-stimulated autophosphorylation through the depletion of ATP, receptor phosphorylation and cellular ATP levels were measured over a range of DNP concentrations. After a 60-min incubation, ATP levels were reduced 50% by 1 mM DNP and >80% by 2 mM DNP (Fig. 2, dashed line). Moreover, the dose-dependent decrease in cellular ATP levels corresponded exactly to the inhibition of insulin-stimulated autophosphorylation detected by immunoprecipitation with αIR (Fig. 2 *Inset* and solid line). The cells were viable during DNP treatment, as 98% excluded 0.1% trypan blue and ATP levels returned to >70% of normal after withdrawal of DNP for 1 hr. These data are consistent with the hypothesis that DNP reduced receptor autophosphorylation by decreasing the intracellular concentration of ATP.

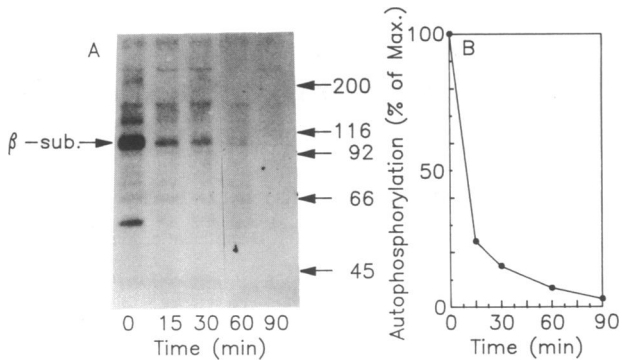

FIG. 1. Time course of inhibition of insulin receptor autophosphorylation by DNP. [^{32}P]Phosphate-labeled Fao cells were incubated with 2 mM DNP at 37°C. (*A*) At the indicated times, the cells were stimulated with insulin, solubilized, immunoprecipitated with αPY, and analyzed by reducing SDS/PAGE and autoradiography. Molecular masses are indicated in kDa. (*B*) The autoradiogram was quantified by scanning densitometry.

Effect of DNP on the Internalization of Insulin and the Insulin Receptor. Shortly after insulin binding to the α-subunit and autophosphorylation of the β-subunit of the insulin receptor, the hormone–receptor complex is internalized by Fao cells (13). The role of autophosphorylation in receptor internalization was tested by measuring the uptake and degradation of insulin following treatment of cells in the absence or presence of 2 mM DNP for 45 min. Cell-associated ^{125}I-insulin in DNP-treated and control Fao cells reached identical steady-state levels within 15–30 min (Fig. 3 *A* and *B*). The accumulation of intracellular insulin in control and DNP-treated cells was relatively low due to the extremely rapid degradation of internalized insulin by Fao cells. However, in DNP-treated and control cells, the amount of internalized ^{125}I-insulin reached steady state with ≈30% of total cell-associated radioactivity in an acid-resistant compartment (Fig. 3*C*). The release of trichloroacetic acid-soluble radioactivity into the medium was also unaffected by DNP. Thus inhibition of insulin-stimulated receptor autophosphorylation by DNP did not inhibit receptor-mediated uptake and degradation of insulin.

To assess insulin-stimulated internalization directly, surface-iodinated cells were incubated without or with DNP (2 mM) for 45 min, stimulated with insulin, and trypsinized to remove surface receptors. The accumulation of internal

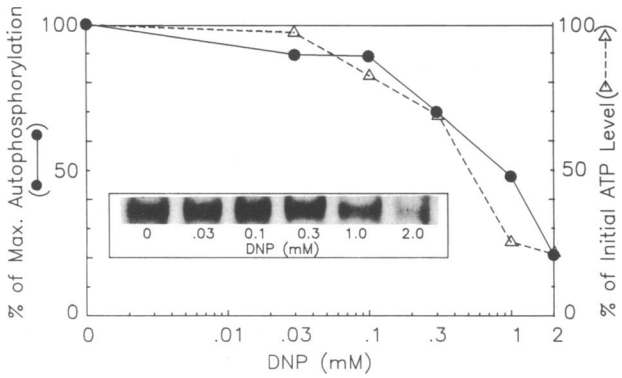

FIG. 2. Inhibition of insulin receptor autophosphorylation and reduction of cellular ATP levels by DNP. ^{32}P-labeled Fao cells were incubated for 45 min with the indicated concentration of DNP at 37°C. The cells were then stimulated with 100 nM insulin, solubilized, and immunoprecipitated with αIR. (*Inset*) Autoradiogram of the insulin receptor β-subunit after SDS/PAGE under reducing conditions. ●, Quantification of the autoradiogram by scanning densitometry. △, ATP levels from cells treated identically were measured with the luciferin/luciferase assay.

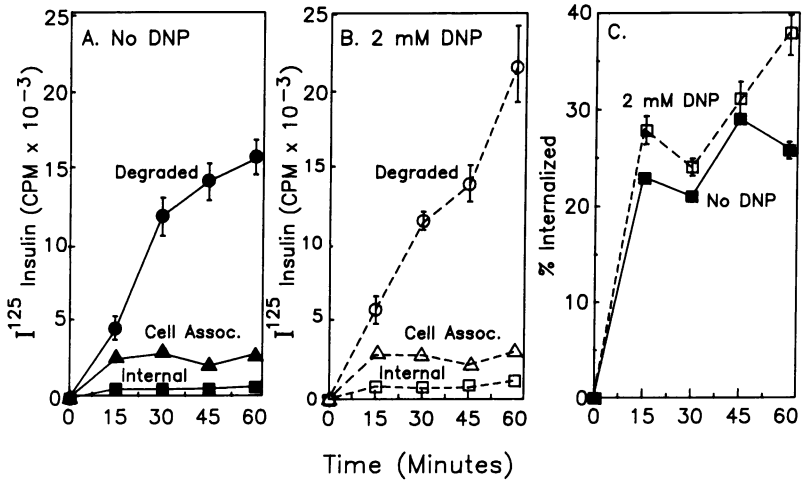

FIG. 3. Internalization and degradation of insulin by Fao cells in the absence or presence of DNP. Fao cells were incubated in the absence (*A*) or presence (*B*) of 2 mM DNP for 45 min. The cells were then incubated with ^{125}I-insulin at 37°C in the continued absence or presence of DNP and at the indicated times washed at 4°C with acidic or neutral PBS to determine the amount of internalized (■, □) or total cell-associated (▲, △) insulin. Aliquots of the media were precipitated with 10% trichloroacetic acid to determine the amount of degraded insulin released by the cells (●, ○). (*C*) The amount of internalized insulin in the absence (■) or presence (□) of DNP was expressed as a percentage of the total cell-associated insulin at each time point.

trypsin-resistant receptors was measured by immunopurification with αIR and analysis by SDS/PAGE (Fig. 4*A*). Insulin receptor internalization was identical in control and DNP-treated cells. To confirm these results, the internalization of insulin receptors in the absence or presence of 2 mM DNP was examined indirectly by measuring the insulin-stimulated reduction in specific cell-surface ^{125}I-insulin binding. After various periods of insulin stimulation at 37°C, surface-bound ligand was removed by an acid wash at 4°C and the residual surface binding capacity was measured with ^{125}I-insulin. In DNP-treated and control cells, insulin (100 mM) caused a 25% reduction in surface binding, reaching steady state within 15–20 min (Fig. 4*B*). Control experiments showed that the acid washing restored insulin binding to >98% of basal levels in cells incubated with insulin at 15°C, a temperature at which internalization in Fao cells is slow (data not shown). The reduction in surface binding after incubations at 37°C was therefore due to translocation of plasma membrane insulin receptors into the cell. Thus no differences in the rate or extent of receptor internalization after DNP treatment were detected, suggesting that receptor internalization and recycling were not impaired by DNP.

DNP Inhibition of Receptor Autophosphorylation Using Iodinated Receptors. The inhibition of insulin-stimulated autophosphorylation of total and internalized insulin receptors was quantified by immunoprecipitation of iodinated receptors using αPY (Fig. 5). This protocol measures the number of tyrosyl phosphorylated receptors rather than their degree of phosphorylation (13). Before DNP treatment, α- and β-subunits of the insulin-stimulated insulin receptor were identified by immunoprecipitation with αPY (Fig. 5, lane a). Although tyrosyl autophosphorylation of the receptor was confined to its β-subunit (22), both subunits were immunoprecipitated with αPY since the $\alpha_2\beta_2$ form of the receptor remains intact during solubilization and immunoprecipitation (13, 23). After incubation of cells with 2 mM DNP, the amount of surface-labeled receptor precipitated with αPY decreased by 80% (Fig. 5, lane f). This reduction in αPY-precipitable iodinated receptors reflects a decrease in the number of tyrosyl phosphorylated receptors.

To determine if the receptors that could still undergo tyrosyl autophosphorylation after DNP treatment were preferentially internalized, we measured the tyrosyl phosphorylation of internalized receptors (Fig. 5). Surface-iodinated cells were stimulated with insulin for varying times and trypsinized to remove surface receptors. After immunopurification with αPY and analysis by SDS/PAGE, internalized receptors were detected by the presence of intact α-subunit bands. The data were derived from the same experiment as that in Fig. 4*B*, which showed identical numbers of αIR-precipitable internalized receptors in control and DNP-treated cells. Internalized tyrosyl phosphorylated receptors were clearly observable in control cells, with an increase in

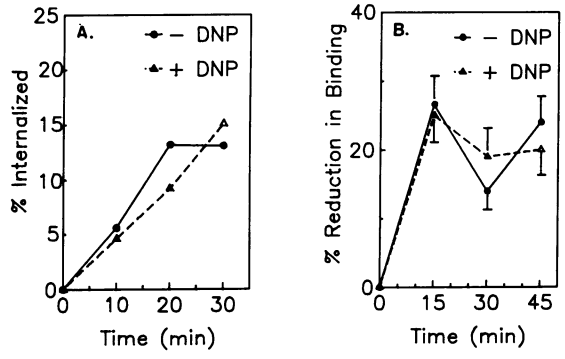

FIG. 4. Insulin-stimulated internalization of the insulin receptor in control and DNP-treated Fao cells. (*A*) Surface-iodinated Fao cells were incubated in the absence (●) or presence (△) of 2 mM DNP for 45 min at 37°C and then stimulated with insulin at 37°C. At the indicated times, the cells were solubilized after incubation for 30 min at 4°C in the absence or presence of trypsin. Proteins were immunoprecipitated with αIR and separated by SDS/PAGE. Internalization was quantified by scanning densitometry of intact α-subunit bands. (*B*) Fao cells were incubated in the absence (●) or presence (△) of 2 mM DNP for 45 min at 37°C and then stimulated with 100 nM insulin at 37°C. At the indicated times the cells were acid-washed at 4°C to remove surface-bound ligand, and surface binding of ^{125}I-insulin during 15 hr at 4°C was determined.

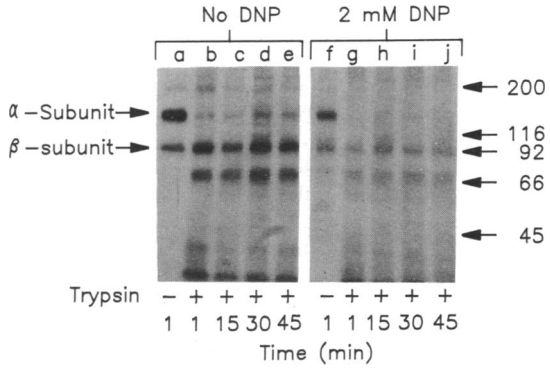

FIG. 5. Inhibition by DNP of autophosphorylation of surface-iodinated insulin receptors. Fao cells were surface-iodinated at 4°C and incubated in the absence (lanes a–e) or presence (lanes f–j) of 2 mM DNP at 37°C for 45 min. The cells were stimulated with 100 nM insulin at 37°C for varying times, incubated for 30 min at 4°C in the absence (lanes a and f) or presence (lanes b–e and g–j) of trypsin, solubilized, and immunoprecipitated with αPY. The immunoprecipitated proteins were separated by SDS/PAGE and visualized by autoradiography. Molecular masses are indicated in kDa.

number after 30–45 min of insulin stimulation (Fig. 5, lanes b–e). In contrast, intact α-subunit bands, reflecting the number of internalized tyrosyl phosphorylated receptors, were barely detectable in DNP-treated cells (Fig. 5, lanes g–j). Furthermore, there was no preferential internalization of the pool of tyrosyl phosphorylated receptors that remained after DNP treatment. Thus, normal rates of insulin and insulin receptor internalization in DNP-treated cells were observed despite marked decreases in the level of whole-cell and internalized receptor tyrosyl phosphorylation. These data suggest that insulin receptor autophosphorylation is not necessary for insulin-stimulated internalization.

DISCUSSION

Our results indicate that internalization and degradation of insulin and recycling of the receptor are independent of β-subunit tyrosyl autophosphorylation. Treatment of Fao cells with 2 mM DNP for 45 min abolishes 80–90% of the insulin-stimulated tyrosine phosphorylation of the receptor but has no effect on insulin uptake or degradation or the rate or extent of insulin-stimulated receptor internalization. Moreover, the tyrosyl phosphorylation of internalized receptors from DNP-treated cells is markedly reduced as compared to control cells. Thus, receptor mobility is normal despite depletion of cellular ATP and inhibition of receptor tyrosyl phosphorylation, suggesting that the endocytosis of the insulin receptor does not require autophosphorylation.

Our data suggest that treatment of Fao cells with DNP reduces the number of tyrosyl phosphorylated insulin receptors rather than the extent of individual receptor phosphorylation. Our interpretation is supported by the fact that an identical reduction in tyrosyl phosphorylation is seen when insulin receptors are immunopurified with αPY from either [^{32}P]phosphate-labeled or surface-iodinated cells. This conclusion is also consistent with *in vitro* studies that indicate that reductions in ATP reduce the number of phosphorylated receptors without affecting the number of phosphorylation sites in the β-subunit (24, 25). Although the αPY used in this study has been shown to recognize receptors phosphorylated on as few as two sites (22), it is not known whether it would detect monophosphorylated receptors. It is unlikely, however, that this receptor species is present in substantial numbers in DNP-treated cells, since immunoprecipitation with αIR and αPY detects similar reductions in β-subunit phosphorylation. Thus DNP treatment causes a reduction in the number of insulin receptors that undergo tyrosyl phosphorylation during insulin stimulation.

The tyrosyl phosphorylation of whole-cell and internalized insulin receptors is markedly reduced by DNP treatment, whereas the number of internalized receptors is unaffected. The 10–20% of receptors that still undergo tyrosyl phosphorylation after DNP treatment do not undergo preferential internalization, in contrast to what would be expected if these receptors mediated the bulk of internalization. These data therefore support the functional independence of receptor autophosphorylation and internalization.

A number of previous studies have suggested that tyrosyl autophosphorylation of cell surface hormone receptors may not be required for ligand-stimulated endocytosis. Fibroblasts from patients with leprechaunism and severe insulin resistance demonstrate normal rates of insulin uptake despite 70–80% decreases in insulin receptor autophosphorylation (26). Similarly, a mutant of the human insulin receptor containing a C-terminal deletion of 95 amino acids is deficient in insulin-stimulated autophosphorylation yet internalizes normally (27). Furthermore, tyrosyl autophosphorylation does not appear to be required for epidermal growth factor (EGF)-stimulated internalization of the EGF receptor. An insertional mutation at residue 708 of the EGF receptor inactivates the tyrosine kinase, but the altered molecule undergoes EGF-stimulated endocytosis (28). Replacement of Lys-721 at the ATP binding site of the EGF receptor has yielded conflicting data, with different groups reporting either impaired internalization or normal internalization but altered postinternalization routing of the receptor (29–31). Tyrosyl phosphorylation is not required for the internalization of non-tyrosine kinase receptors such as the low density lipoprotein and insulin-like growth factor II/mannose 6-phosphate receptors, which mediate endocytotic uptake of their ligands (32–34). However, tyrosyl phosphorylation of the insulin receptor may influence its intracellular trafficking once internalization has occurred. Treatment of rat adipocytes with vanadate increases insulin receptor autophosphorylation *in vivo* and increases the proportion of internalized insulin that is routed to a degradative pathway (35, 36). No differences in the degradation of insulin were detected in DNP-treated Fao cells during our studies.

Mutagenesis of the insulin receptor by replacement of Lys-1018 alters the ATP binding site and abolishes receptor autophosphorylation and insulin-stimulated internalization in Chinese hamster ovary cells (15–17). These results suggest that internalization, like other biological responses, is mediated by autophosphorylation of the β-subunit. The discrepancy between the data on internalization of the Lys-1018 mutant and our results may be cell-type specific. However, the inhibition of receptor internalization by replacement of Lys-1018 may be due to conformational changes in the β-subunit that alter its recognition by the endocytotic machinery. Alternatively, the Lys-1018 mutation might interfere with insulin-induced clustering of the receptors in coated pits. In this regard, it is interesting to note that internalization of the mutant receptor was stimulated by a divalent monoclonal antibody (15). Finally, the inability of the Lys-1018 mutant receptor to internalize may be related to its diminished serine phosphorylation *in vivo*. Serine phosphorylation of the β-subunit has been correlated with a stimulation of insulin receptor internalization in phorbol ester-treated cells (37, 38), and insulin-stimulated changes in serine phosphorylation of other growth factor receptors have been shown to correspond to changes in their intracellular redistribution (39).

Previous studies have shown that the uptake of insulin and other ligands (18, 40, 41) as well as internalization of the insulin receptor (42, 43) occur in cells despite the depletion of cellular ATP by energy poisons, whereas the biological activity of the insulin receptor is blocked under comparable conditions (44). The 80% reduction in cellular ATP levels by DNP seen here is similar to that observed by others (42). The preferential inhibition by DNP of autophosphorylation without affecting internalization probably relates to favorable differences in the binding affinities for ATP in each process and does not suggest that internalization is independent of metabolic energy. Although the K_m of the insulin receptor for ATP is about 25 μM *in vitro*, this value is probably much higher *in vivo* as the cytosolic Mn^{2+} concentration is very low (24). Several known ATP-dependent steps in the endocytic pathway have been identified, most notably the ATP-dependent proton pump identified in coated vesicles and the uncoating ATPase (45, 46). The latter enzyme has an extremely low K_m for ATP under *in vivo*-like conditions and might reasonably be expected to function adequately at reduced cellular ATP levels (18).

The independence of insulin receptor autophosphorylation and internalization in DNP-treated cells strongly suggest that the two functions of the receptor are not causally linked *in vivo*, whereas mutation of the ATP binding site of the receptor suggests that they are related (15–17). Caution must be used in extrapolating from results obtained with pharmacologically treated cells or cells transfected with mutant receptors, as in either case a single perturbation may have

multiple effects. Therefore, we cannot rule out the possibility that DNP induces changes in the phosphorylation or turnover of some protein that would inhibit the internalization of unphosphorylated receptors in the absence of DNP. Nonetheless, this possibility is consistent with our suggestion that tyrosyl autophosphorylation is not the principal regulator of insulin receptor internalization.

In summary, we have uncoupled insulin-stimulated autophosphorylation of the insulin receptor from insulin-stimulated endocytosis. DNP treatment of Fao cells markedly inhibited receptor tyrosine phosphorylation without diminishing receptor internalization or insulin uptake and degradation. These data suggest that tyrosyl autophosphorylation of the insulin receptor and activation of the tyrosine kinase are not required for internalization.

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