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Overexpression of Insulin Degrading Enzyme: Cellular Localization and Effects on Insulin Signaling

Karen A. Seta and Richard A. Roth[1]
Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California 94305

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To investigate the role of insulin degrading enzyme (insulysin, EC 3.4.24.56) in insulin signaling, Chinese hamster ovary cells overexpressing the human insulin receptor were genetically engineered to also stably overexpress the rat insulin degrading enzyme. In comparison to the parental cells, these cells expressed 2.7-fold elevated levels of enzyme and insulin degradation was also increased 2-fold. These cells also exhibited a more rapid decrease in receptor tyrosine phosphorylation after removal of insulin. Moreover, low concentrations of insulin were less effective at stimulating proliferation of the cells overexpressing the enzyme. Finally, a fraction of the overexpressed enzyme as well a fraction of the endogenous enzyme could be detected on the plasma membrane surface of these cells. These results support the hypothesis that this enzyme may function in insulin signaling by degrading the insulin molecule.

After insulin binds to its specific plasma membrane receptor, it activates a cascade of signaling molecules which subsequently stimulate a variety of biological responses (1). Each cell that responds to the hormone must also have a mechanism for terminating this response. One method for accomplishing this is to degrade the hormone molecule itself. Indeed, insulin is rapidly degraded by every cell that responds to the hormone (reviewed in refs. 2-4). Despite extensive studies over many years, the exact mechanism whereby cells degrade the insulin molecule is still not known. One enzyme that has been proposed to play a role in this process is a specific metalloprotease which has been called insulin degrading enzyme (IDE), insulinase and most recently, insulysin (2-5). A variety of data have supported this hypothesis including, most recently, the finding that overexpression of this enzyme can increase the rate of degradation of insulin by intact cells (6). Unfortunately, stable overexpression of this enzyme has, so far, only been reported in KMHL2 cells, a line of cells with almost no detectable insulin receptors (IR) and insulin responses (6).

In the present studies we therefore sought to develop a stable cell line which contains both the insulin receptor as well as overexpressed IDE. To this end, Chinese hamster ovary cells expressing the human IR (CHO-IR) (7) were transfected with a clone encoding the rat IDE (8, 9) and colonies expressing elevated levels of the enzyme were identified. These cells were then compared with the non-overexpressing parental cells for their ability to degrade insulin and respond to the hormone. In addition, a fraction of the endogenous and overexpressed enzyme was shown to be labeled by a membrane-impermeable biotinylating agent, indicating that a portion of IDE is present on the extracellular surface of the cells. This cell surface enzyme may participate in the degradation of the insulin molecule.

MATERIALS AND METHODS

Materials. Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and tissue culture reagents were purchased from the University of California, San Francisco, Cell Culture Facility. CHO cell lines were maintained in Ham's F-12 supplemented with 10% newborn calf sera and 1% penicillin-streptomycin.

Transfection of CHO-IR cells. Four μg of the rat IDE cDNA (engineered to lack the first AUG) in the SRα mammalian expression vector (10) and 10 ng of pSV2.puro vector (11) were cotransfected into CHO-IR cells using the calcium phosphate precipitation procedure. Individual clones were selected and expanded in medium supplemented with 10 μg/ml puromycin. Screening for overexpression was accomplished by Western blotting of cell lysates with the anti-IDE antibody, 9B12 (12). Those clones expressing the highest amount of IDE were chosen for further expansion and analysis. The same blots were also probed with a polyclonal anti-IR antibody to ensure that the selected clones expressed the same amount of IR as the parental CHO-IR cells.

Insulin degradation assays. CHO, CHO-IR-IDE and CHO-IR cells in 24-well dishes were washed once with complete medium, placed on ice and then 280 μl complete growth medium containing 5D9 (13) or control Ig was added. After 30 min at 37°C, 100 pM ^{125}I-insulin (final concentration) (Linco Research, St. Charles, MO) was

[1] To whom correspondence should be addressed. Fax: 415-725-2952. E-mail: roth@cmgm.stanford.edu.

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added and the cells were incubated for an additional 30 min at 37°C. The reaction was stopped by placing the plates on ice, removing the medium and adding 1 mM N-ethyl-maleimide, an inhibitor of insulin degradation. The intactness of the insulin was assessed by a receptor binding assay (4) in which the source of IR was rat liver so that the presence of 5D9 would not effect receptor binding. Controls of ^{125}I-insulin diluted in media were utilized to assess 100% binding (i.e., 0% degradation). Percent degradation was calculated as follows: [1 − (cpm bound in the sample / cpm bound in the control)] × 100.

Insulin receptor autophosphorylation assay. A colorimetric assay was utilized as described (14). In brief, cells in 6-well tissue culture dishes were incubated with Ham's F-12 with 20 mM HEPES, pH 7.6, and 1 mg/ml BSA for 1 hour at 37°C. Insulin was added to a final concentration of 10 nM and cells were incubated for 1 hour at 4°C. The media was then replaced with fresh media minus insulin, and the cells were incubated at 37°C for various times. Cells were then placed on ice and lysed in buffer with phosphatase inhibitors (50 mM HEPES, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mg/ml bacitracin, 1 mM Na orthovanadate, 20 mM $NaPP_i$, and 20 mM NaF). Aliquots of lysates were incubated overnight at 4°C with microtiter wells that had been previously coated with the anti-IR antibody 29B4. Wells were then incubated for 2 hours at 22°C with either a biotinylated anti-phosphotyrosine antibody (UBI) or a biotinylated anti-IR antibody (2G7) (13) in a buffer containing BSA and phosphatase inhibitors. Washed wells were then incubated with streptavidin conjugated to horseradish peroxidase followed by TMB One-Step colorimetric substrate. The color reaction was stopped by adding an equal volume of 4 N H_2SO_4, and the absorbance at 490 nm was read on a microplate reader (BioRad Model 450).

Cell proliferation assay. Cells were trypsinized and resuspended in Ham's F12 with 20 mM HEPES, pH 7.6 and 10 mg/ml BSA. Five thousand cells/well were plated in 96-well tissue culture dishes in 50 μl media and allowed to recover for 12 hours. Various concentrations of insulin were then added, and cells were grown for an additional 48 hours. At that time the number of viable cells was determined by a colorimetric assay, the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay Kit (Promega Corp., Madison, WI) according to the manufacturer's directions. Absorbance at 490 nm was read with a microplate reader (BioRad Model 450). As a control, maximal stimulation of proliferation was achieved by plating 2500 cells/well and adding medium containing 1% newborn-calf serum at the same time that insulin was added to other samples.

Cell surface biotinylation. Cell surface proteins were biotinylated by a modification of the method of Levy-Toledano (15). Briefly, cells were placed on ice and washed 3 times with ice-cold PBS-CM (phosphate buffered saline, pH 7.6, with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$). Biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester (final concentration of 0.5 mg/ml) was added and the cells were incubated at 4°C for 5 min. The reaction was quenched by washing cells 3 times in ice-cold 20 mM Tris-Cl, pH 7.6/ 120 mM NaCl. Cells were lysed and lysates were immunoprecipitated with monoclonal antibodies as described (16). Immunoprecipitates were boiled in Laemmli sample buffer (17) and analyzed by SDS-PAGE. Proteins were then transferred to nitrocellulose and biotin-labeled proteins were detected with ExtrAvidin- Alkaline Phosphatase according to the manufacturer's directions.

RESULTS

Overexpression of IDE and effect on insulin degradation. Since prior attempts to obtain CHO-IR cells stably expressing increased total IDE levels with a human IDE cDNA had failed (18), a cDNA encoding the rat enzyme was utilized. In addition, because both human and rat IDE contain an upstream AUG codon (9, 19) which can hinder translation at the downstream start site (20), the cDNA was altered to remove this region. This alteration was found to allow for a higher level of IDE expression without any change in the properties of the expressed protein (data not shown). CHO-IR cells were cotransfected with the IDE construct and a plasmid encoding puromycin resistance, drug selected and the resulting colonies screened for levels of total IDE by immunoblotting. Cells were picked which had at least a 2-fold increase in total IDE levels and the same levels of IR as the original cells (Fig. 1A).

To measure the ability of these cells to degrade insulin, intact cells in complete media were incubated with 100 pM of monoiodinated insulin. After 30 min at 37°C, the media was removed and the intactness of the insulin was assessed in a receptor binding assay. Insulin from the cells overexpressing IDE exhibited approximately twice the extent of degradation as the insulin from the non-IDE overexpressing cells (49±2% versus 24±2%) (Fig. 1B). As expected, CHO cells which did not express the human insulin receptor exhibited even lower levels of insulin degradation (less than 5%) (Fig. 1B), verifying that in this system most of the insulin degradation occurs via a receptor mediated process.

To determine whether the increase in insulin degradation observed in the CHO-IR cells overexpressing IDE was also occuring via a receptor mediated process, insulin degradation mediated by these cells was also assessed in the presence of a monoclonal antibody (5D9) which blocks the binding of insulin to the human receptor (13). This antibody inhibited by 86% and 99% the degradation of insulin observed with cells overexpressing IDE and the non-overexpressors, respectively (Fig. 1B).

Effect of IDE overexpression on IR autophosphorylation and insulin-stimulated mitogenesis. To deter-

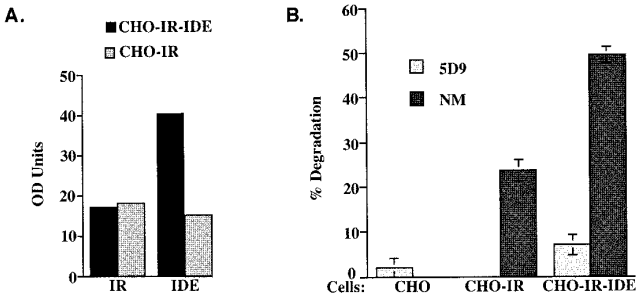

FIG. 1. Characterization of CHO cells overexpressing IDE. A. Levels of IDE and IR in CHO-IR and CHO-IR-IDE. Cells were lysed and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with either α-IR or α-IDE antibodies. Blots were scanned and quantified using the NIH Image program. B. Insulin degradation by CHO, CHO-IR and CHO-IR-IDE. Cells were incubated with either 100 nM control Ig or monoclonal anti-IR antibody 5D9 as indicated for 30 min at 37°C. Then 100 pM of ^{125}I-insulin was added and after an additional 30 min the extent of insulin degradation was assessed by a radioreceptor assay.

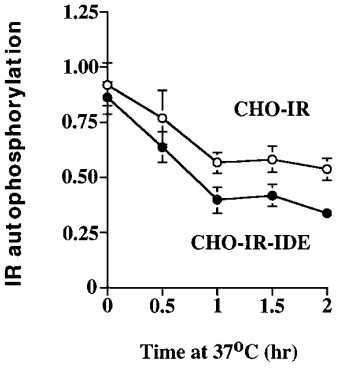

FIG. 2. Effect of overexpression of IDE on IR autophosphorylation. CHO-IR and CHO-IR-IDE cells were preincubated with 10 nM insulin for 1 hour at 4°C, washed and then the cells were incubated at 37°C for the indicated times. The decay of the IR autophosphorylation signal was assessed by ELISA. The level of tyrosine phosphorylation is normalized to the amount of insulin receptor, and the results shown are the mean ± s.e.m. of three experiments. Where not shown, error bars are too small to be seen.

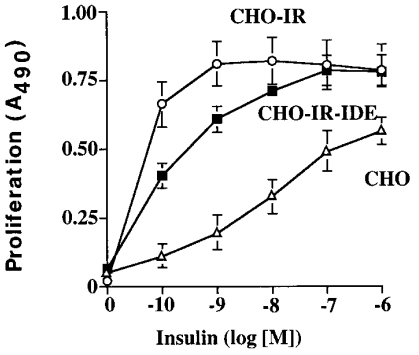

FIG. 3. Effect of overexpression of IDE on insulin-stimulated proliferation. CHO, CHO-IR, and CHO-IR-IDE cells were grown in 96-well tissue culture dishes in the presence or absence of the indicated concentrations of insulin. The number of viable cells was determined after 48 hours by a non-radioactive colorimetric assay. The results shown are the mean ± s.e.m. of three experiments, each done in duplicate.

mine whether IDE overexpression effected subsequent IR signaling, we first examined IR autophosphorylation. Cells were treated for 1 hour at 4°C with 10 nM insulin to maximally stimulate IR autophosphorylation. The cells were then washed and the extent of IR tyrosine phosphorylation was measured after various periods of time at 37°C to allow for insulin degradation and IR dephosphorylation. Cells overexpressing IDE exhibited a more rapid decrease in receptor tyrosine phosphorylation than the cells not overexpressing IDE (Fig. 2), consistent with a more rapid degradation of insulin by these cells.

To determine whether overexpression of IDE would effect the ability of insulin to stimulate a biological response in these cells, we utilized the ability of insulin to stimulate a proliferative response in CHO-IR cells. Cells were incubated with various concentrations of insulin for 48 hours and then the number of viable cells was determined by a colorimetric assay for dehydrogenases. The proliferative response in the cells overexpressing IDE was most greatly effected at the lower concentrations of insulin; at 100 pM insulin the response in the cells overexpressing IDE was 0.40±0.05 versus the 0.66±0.08 in the cells not overexpressing IDE. The maximal level of stimulation of proliferation of these two cell lines with either 1 μM insulin or 1% serum were approximately the same, indicating that overexpression of IDE did not have a non-specific effect on their proliferative capacities. As previously reported (7), CHO cells which did not overexpress the IR also had a decreased proliferative response to insulin (Fig. 3).

Surface localization of IDE. To determine whether some of the expressed IDE could be on the plasma membrane, cells were incubated with a membrane-impermeable biotinylating reagent (biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester), washed, lysed and the IDE was immunoprecipitated. The presence of biotinylated IDE was detected by blotting the immunoprecipitated IDE with streptavidin-alkaline phosphatase. A band of the correct molecular mass for IDE was clearly observed in the anti-IDE immunoprecipitates from the cells overexpressing IDE but not in the control Ig precipitates (Fig. 4). A fainter but still detectable IDE band was also observed in the anti-IDE precipitates from the cells not overexpressing IDE, presumably the endogenous IDE. Controls verified the labeling of the cell surface IR but not a cytoplasmic protein, phosphatidylinositol 3-kinase (Fig. 4). Cell sur-

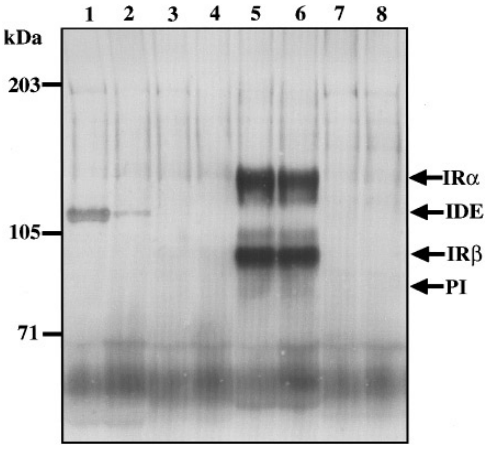

FIG. 4. Identification of cell surface IDE by biotinylation of CHO-IR and CHO-IR-IDE Cells. Cells were biotinylated, lysed, and the lysates were immunoprecipitated with either antibodies to IDE (Lanes 1-2), normal mouse IgG (Lanes 3-4), antibodies to the IR (Lanes 5-6), or antibodies to the cytosolic protein phosphatidylinositol 3-kinase (Lanes 7-8). The immunoprecipitates were analyzed by SDS-PAGE and blotted with avidin coupled to alkaline phosphatase. Odd lanes, CHO-IR-IDE cells; even lanes, CHO-IR cells.

face IDE could also be detected on a variety of other cells including the previously described KMHL2 cells overexpressing IDE (6) as well as in kidney cell lines not overexpressing IDE such as the COS cells and the Madin-Darby canine kidney cells (MDCK). In the latter cells, approximately 2 to 3 times more IDE was observed on the apical membranes than on the basolateral membranes, in agreement with prior studies indicating that the apical membranes of kidney cells are more active at degrading insulin (21). The biotinylation of IDE was extremely rapid, being detectable even after only 1 min of reaction. In addition, biotinylation was detecable with as little as 0.03 mg/ml of the biotinylating reagent, indicating that it was likely that a cell surface molecule was being labeled.

DISCUSSION

IDE is a metalloprotease which is representative of a family of highly evolutionarily conserved enzymes with homologous molecules being present in bacteria, Drosophila and yeast (4, 5, 22). In the latter, the enzyme has been implicated in bud site slection and propheromone processing of one of the yeast mating factors (22). The physiological role of the mammalian enzyme has been more controversial. Since the enzyme readily cleaves insulin, it has been proposed to play a role in the degradation of this hormone (2-4). However, the sequence of the mammalian enzyme contains a carboxy-terminal peroxisome targeting signal, suggesting that this enzyme may be peroxisomal (9). Indeed, fractionation studies have indicated that approximately 30% of the enzyme is peroxisomal with the remainder being cytosolic (23). Other studies have suggested that some of the enzyme may be localized in the membrane of cells (2).

One of the problems in studying the physiological role of IDE has been the difficulty in obtaining cell lines which express increased amounts of this protein. One study has obtained a stable line of cells which contained an inducible IDE (6). These cells did exhibit increased rates of insulin degradation after the induction of IDE (6). However, due to the low levels of IR in these cells, it was not possible to study the effect of the increased levels of IDE on insulin signaling. In the present studies we have been able to produce a line of CHO-IR which exhibits an approximate 2.7-fold increase in total IDE levels, assuming that the monoclonal anti-IDE antibody utilized to measure IDE levels equally recognizes the endogenous hamster IDE and the expressed rat IDE. There was a corresponding approximate 2-fold increase in insulin degradation in these cells. This increased rate of insulin degradation was, to a large extent, still receptor mediated. Moreover, the increase in IDE levels in these cells was accompanied by a more rapid decrease in IR autophosphorylation after removal of the insulin, consistent with the increase in insulin degrading ability of these cells. Finally, the cells overexpressing IDE also exhibited a decreased ability to respond to low levels of insulin in a proliferative response, again consistent with the increased ability of these cells to degrade insulin. The lack of difference in the proliferative response of these cells to high concentrations of insulin and to serum indicates that overexpression of IDE did not have a general effect on the proliferative capacity of the cells.

The finding of a fraction of both the endogenous IDE as well as the overexpressed enzyme on the extracellular surface of the CHO cells is consistent with prior studies suggesting the presence of some surface IDE in other cell types (24, 25). However, these prior studies utilized a polyclonal antibody which is now known to recognize additional proteins. In the present work surface IDE was clearly identified on the surface of both the CHO cells overexpressing IDE as well as the cells not overexpressing this enzyme. The rapid labeling of the enzyme with a membrane impermeable biotinylation reagent as well as the lack of labeling of a cytosolic protein in the same experiment clearly argue for a cell surface localization of some of the IDE molecules. We also observed this surface labeling of IDE in several other cell types including two kidney cell lines (COS and MDCK) which were not overexpressing IDE. From the amino acid sequence of IDE, it is not clear how this molecule would be routed to the plasma membrane (9, 19). Attempts to obtain increased levels of surface IDE by incubating intact CHO cells with extracts of cells overexpressing IDE were unsuccessful, arguing that it was unlikely that the surface IDE was coming from IDE leaking into the media. Interestingly, a number of proteins lacking a classical hydrophobic signal peptide sequence (including yeast mating factor, a 110 kDa hemolysin, and fibroblast growth factor) have also been previously reported to be directed to the extracellular environment, possibly via specific membrane transporters in the ATP-binding cassette family (26, 27). It is possible that IDE utilizes one of these pathways to be delivered to the extracellular surface of cells. This surface IDE could participate in the insulin degradation process, possibly via its internalization with the IR into endosomes.

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