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EFFECTS OF METALLOENDOPROTEASE INHIBITORS ON INSULIN BINDING, INTERNALIZATION AND PROCESSING IN ADIPOCYTES

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SUMMARY: The effects of metalloendoprotease inhibitors on insulin binding, internalization, and processing were studied in isolated rat adipocytes. The metalloendoprotease inhibitor phosphoramidon caused a marked (threefold) increase in intracellular insulin accumulation without affecting surface binding. The dipeptide metalloendoprotease substrate analogues benzyloxycarbonyl-Gly-Phe-NH_2 and benzyloxycarbonyl-Gly-Leu-NH_2 caused similar large increases in intracellular insulin but also caused a doubling of cell surface bound insulin. The effect on surface binding was due to increased insulin receptor affinity as demonstrated by Scatchard analysis and the benzyloxycarbonyl-Gly-Phe NH_2 induced inhibition of the dissociation of prebound insulin from the cell surface. These results suggest a role for endogenous metalloendoprotease-like enzymes in insulin processing by rat adipocytes.

An approach for investigating the role of proteases in mediating various cellular processes involves examining the effects of the appropriate protease inhibitors on the particular cellular effect under consideration. Examples of this include the use of different protease substrate analogues in examining the role(s) of proteolytic steps in insulin mediator generation (1), insulin stimulated glucose transport and metabolism (2,3), and insulin receptor phosphorylation (4).

Recently, a role for metalloendoproteases has been implicated in cellular events dependent upon membrane fusions and vesicular movements. Thus, inhibitors of metalloendoproteases have been shown to block the exocytotic release of histamine (5), catecholamines (5), and neurotransmitters (6) as well as the fusion of myoblasts into myotubules (7). Since the endocytotic uptake and intracellular processing of cell surface bound insulin similarly requires vesicular movements and multiple membrane

Abbreviations: Z-, benzyloxycarbonyl; Hepes, N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid.

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fusion events, we studied the effects of metalloendoprotease inhibitors on the internalization and intracellular processing of receptor-bound insulin.

MATERIALS AND METHODS: Porcine monocomponent insulin was supplied by Dr. Ronald Chance and A_{14}-^{125}I-monoiodo-insulin by Dr. Bruce Frank of Eli Lilly Co., Indianapolis, IN. Collagenase was purchased from Worthington Biochemical Corp. (Freehold, N.J.), Eagles Minimal Essential Medium from Gibco (Grand Island, N.Y.), bovine serum albumin (BSA, Fraction V) from Armour Pharmaceutical Co. (Chicago, IL.), silicone oil from Arthur H. Thomas Co. (Philadelphia, PA), Z -Gly-Phe-NH_2, Z-Gly-Leu-NH_2, Z-Gly-Gly-NH_2, Z-Gly-Ser-NH_2, and Z-Thr-Gly-NH_2 from Vega Biochemicals (Tucson, AZ), phosphoramidon from Enzyme Systems Products (Livermore, CA), and dimethyl sulfoxide and Hepes from Sigma (St. Louis, MO.).

Adipocyte Preparation and Measurement of Total Cell Associated, Surface Bound and Intracellular ^{125}I-Insulin: Isolated adipocytes were prepared by collagenase digestion (8) of epididymal fat pads obtained from male Sprague-Dawley rats weighing 200-240 grams. The cells were filtered through a 250 micron nylon mesh, washed four times and suspended in binding buffer (pH 7.6) consisting of minimal essential medium, 10 mM Hepes and 1% BSA. Adipocyte counts were performed by a modification (9) of Method III of Hirsch and Gallian (10). For measurement of total cell associated insulin, ~400,000 cells/ml were incubated in the binding buffer with 0.3 ng/ml ^{125}I-insulin in the presence or absence of the various protease inhibitors. In all studies, parallel control incubations were performed in the absence of the inhibitors. The dipeptide substrate compounds were first dissolved in dimethyl sulfoxide resulting in a final solvent concentration of 1% in the incubation mixture. Although 1% dimethyl sulfoxide was found to have no effect on insulin binding, internalization or degradation, equal amounts of the solvent were included in all parallel control incubations. At the indicated times, three 200 ul aliquots were withdrawn from the incubations and total cell associated radioactivity measured after separation of the cells by centrifugation through silicone oil (9,11). Since total cell associated ^{125}I-insulin at 37°C represents both surface (receptor) bound and internalized insulin, the amount of ^{125}I-insulin in each of these cellular compartments was determined using a modification (9,11) of an acid extraction procedure (12) which rapidly and quantitatively removes surface bound insulin leaving behind intracellular insulin (9,11). By this method, the acid-extractable ^{125}I-material represents ^{125}I-insulin bound to cell surface receptors while the nonextractable material represents insulin that has been internalized. All data for total, nonextractable and extractable binding were corrected for nonspecific binding measured in parallel incubations in the presence of 50 µg/ml unlabeled porcine insulin. Statistical analysis was performed using Student's t test for paired data since in each experiment parallel incubations were performed in the presence or absence of the various agents.

RESULTS AND DISCUSSION

The substrate specificity of a given class of proteases is determined by the types of adjacent amino acid residues whose peptide bonds are susceptible to hydrolysis by that class of proteases (13). The substrate specificity of metalloendoproteases is such that the amino group of the peptide bond being hydrolyzed is contributed by a large aliphatic amino acid such as leucine or by a nonpolar aromatic amino acid such as phenylalanine (14,15). Thus, the synthetic dipeptide amides, Z-Gly-Leu-NH_2 and Z-Gly-Phe-NH_2 fulfill this structural requirement and have been well characterized as substrates for metalloendoproteases (14,15). Such enzymes are also sensitive to inhibition by phosphoramidon, a bacterial derived protease inhibitor (16).

Table I

Effects of Substrate and Nonsubstrate Z-Dipeptide Amides, Phosphoramidon, and Chloroquine on Insulin Binding and Processing by Rat Adipocytes

Addition	Cell Associated Insulin, Percent of Control		
	Total	Nonextractable (Intracellular)	Extractable (Surface Bound)
Control	100	100	100
a. phosphoramidon (.1mM)	202±15*	301±21*	107±6
b. chloroquine (.1 mM)	206±6*	309±30*	106±18
c. Z-Gly-Phe-NH_2 (5 mM)	270±24*	354±40*	199±24*
Z-Gly-Leu-NH_2 (7 mM)	213±13*	227±8*	201±27*
d. Z-Gly-Gly-NH_2 (5 mM)	118±4	124±3	115±5
Z-Thr-Gly-NH_2 (5 mM)	108±3	112±7	106±2
Z-Gly-Ser-NH_2 (5 mM)	109±2	116±4	104±5

Adipocytes were incubated for 60 min at 37°C with 0.3 ng/ml of ^{125}I-insulin either in the presence or absence of the various agents. Total, surface bound and intracellular insulin were then quantitated. Results shown represent the mean ± SEM of four separate experiments.

*$p<0.001$ compared to control cells.

Table I shows the effects of phosphoramidon, dipeptide metalloendoprotease substrate analogues, and nonsubstrate dipeptide amides on insulin binding and intracellular accumulation. The effect of chloroquine was also studied in parallel because of its known inhibitory action on intracellular degradation of insulin (9,11,17) and its receptor (18,19). Phosphoramidon caused a threefold increase in intracellular insulin accumulation during the one-hour incubation at 37°C without affecting surface binding. This effect was very similar to that observed in the presence of chloroquine alone. The metalloendoprotease substrate analogues Z-Gly-Phe-NH_2 and Z-Gly-Leu-NH_2 both markedly increased total cell associated insulin but, in contrast to the results obtained with phosphoramidon or chloroquine, this was due to increases in both cell

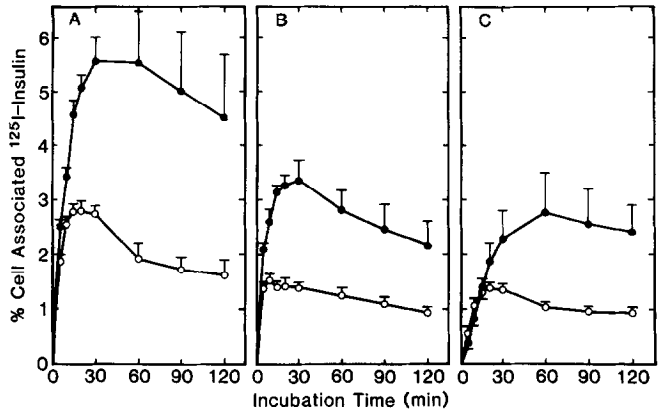

FIGURE 1. **Time course of the effect of Z-Gly-Phe-NH_2 on cell associated total, extractable and nonextractable ^{125}I-insulin.** Adipocytes were incubated at 37°C with 0.3 ng/ml of ^{125}I-insulin in the absence (○) or presence (●) of 5mM Z-Gly-Phe-NH_2. At the indicated times, aliquots of the cells were removed in triplicate for measurement of total (A), surface bound (B) and intracellular (C) cell associated radioactivity. Each data point represents the mean ± SEM of the percent ^{125}I-insulin specifically associated with 4 x 10^5 adipocytes in three separate experiments.

surface bound and intracellular insulin (Table I). Dose-response studies showed that these effects were maximal at 5mM Z-Gly-Phe-NH_2 and 7 mM Z-Gly-Leu-NH_2 with higher concentrations causing increases in nonspecific binding (data not shown). To determine the specificity of the metalloendoprotease substrate analogue Z-dipeptide amides in increasing surface binding and intracellular accumulation of insulin, the effects of three nonsubstrate Z-dipeptide amides were also examined. As shown in Table I, Z-Gly-Gly-NH_2, Z-Thr-Gly-NH_2 and Z-Gly-Ser-NH_2 all of which lack the structural requirements of metalloendoprotease substrates (14,15), produced only small changes in total, nonextractable or extractable cell associated insulin. These results demonstrate that the effect of Z-dipeptide amides in increasing surface bound and intracellular insulin is specific for those Z-dipeptide-amides that are substrates for metalloendoproteases and is not shared by nonsubstrate Z-dipeptide amides. Such results indicate that the observed effects of the substrate compounds are not due to nonspecific cellular perturbation or toxicity.

To further characterize the increase in cell-associated insulin induced by the metalloendoprotease substrate analogues, the effects of Z-Gly-Phe-NH_2 were studied in more detail. Figure 1A shows that the effect of Z-Gly-Phe-NH_2 in increasing total cell associated insulin was rapid, being observed within 5 minutes and becoming maximal (>2-fold increase) by 30 minutes of incubation. At all time points, cell surface bound

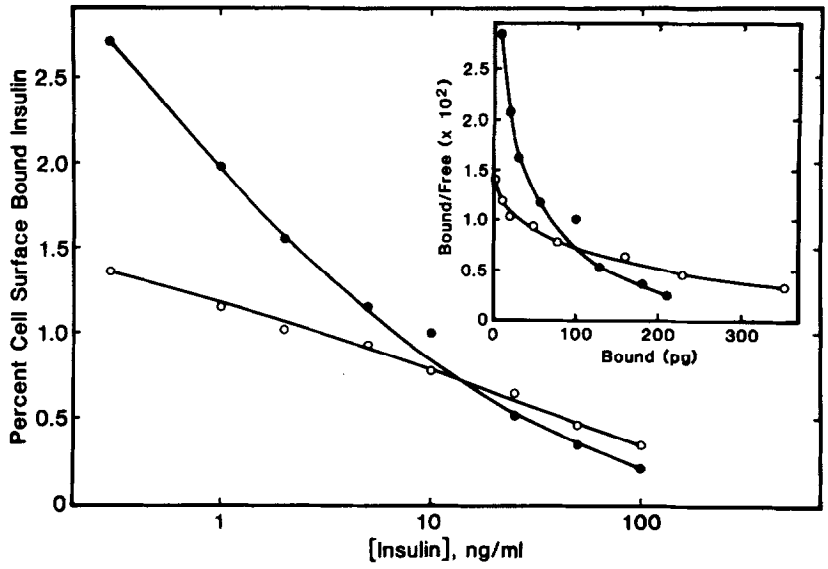

FIGURE 2. **Competition curves and Scatchard plots of ^{125}I-insulin binding to control and Z-Gly-Phe-NH_2 treated cells.** Adipocytes were incubated at 37°C with 0.3 ng/ml of ^{125}I-insulin (plus unlabeled insulin giving the indicated final concentrations) either in the absence (○) or presence (●) of 5mM Z-Gly-Phe-NH_2. After 30 minutes, surface bound insulin was determined in triplicate. The Scatchard plot shown in the inset was derived from the competition curve. The data shown are the means of three separate experiments and represent the amount of specifically cell associated ^{125}I-insulin per $4x10^5$ adipocytes.

insulin was increased in the presence of Z-Gly-Phe-NH_2 (Fig. 2B). By contrast, analysis of the intracellular insulin (Fig. 2C) shows that differences between control and Z-Gly-Phe-NH_2 treated cells did not become apparent until about 20 minutes of incubation. The initial rate of insulin internalization was identical in cells from control and treated incubations (comparing intracellular insulin at 5, 10, 15 minutes in Fig. 2C). Therefore, the increased amounts of intracellular insulin at later time points are most likely a consequence of inhibition of intracellular insulin processing rather than a consequence of increased internalization secondary to increased surface binding.

The increase in cell surface insulin binding induced by the metalloendoprotease substrates (Table I, Fig. 1B) could be due to either an increase in insulin receptor number or affinity. To differentiate between these possibilities, competition curves of surface bound (extractable) ^{125}I-insulin were generated in the presence or absence of 5mM Z-Gly-Phe-NH_2 (Fig. 2). Incubations were performed at 37°C because at low temperatures such as 16°C, treatment of adipocytes with Z-Gly-Phe-NH_2 caused large and inconsistent increases in nonspecific binding (possibly due to precipitation of the

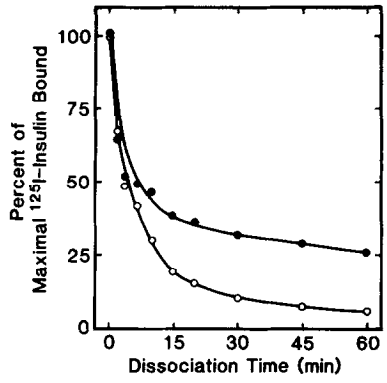

FIGURE 3. **Dissociation of ^{125}I-insulin from control and Z-Gly-Phe-NH_2 treated cells.** Adipocytes were incubated with 0.6 ng/ml of ^{125}I-insulin at 12°C. After 60 minutes, the cells were washed and resuspended in insulin-free medium either in the absence (○) or presence (●) of 5mM Z-Gly-Phe-NH_2. The incubations were then warmed to 37°C to allow dissociation of the bound insulin. At the indicated times, equal (200 µl) aliquots of cells were removed and specific cell-associated ^{125}I-insulin measured. Results shown are representative of three separate experiments.

agent). The data in Fig. 2 demonstrate that the Z-Gly-Phe-NH_2 induced increases in surface binding were seen primarily at the lower insulin concentrations. Scatchard plots (20) of surface bound insulin were both curvilinear and consistent with an increased affinity of the insulin receptor in the presence of Z-Gly-Phe-NH_2 (Fig. 2). The data do not indicate an increase in receptor number and in fact an apparent decrease in surface receptor number was observed in the presence of Z-Gly-Phe-NH_2 (Fig. 2). This may be the result of inhibiting processing and recycling (19,21) of internalized insulin receptors thereby causing their intracellular trapping. Further evidence that the metalloendoprotease substrate analogues are increasing surface binding by increasing insulin receptor affinity is shown in Fig. 3 which demonstrates that dissociation of insulin prebound to the cell surface at 12°C was markedly slowed in the presence of Z-Gly-Phe-NH_2, consistent with an increase in receptor affinity.

The results presented in this study show that metalloendoprotease inhibitors markedly increase intracellular insulin accumulation in rat adipocytes. The effects of phosphoramidon were quantitatively similar to those of chloroquine, the "lysosomotropic" drug whose effects on the processing of internalized insulin have been well documented (9,17). Since phosphoramidon is a potent inhibitor of metalloendoproteases (16), it presumably increases intracellular insulin by inhibiting an endogenous adipocyte metalloendoprotease. This protease activity could be involved in one of the many membrane fusion events necessary for insulin internalization and processing or could

be directly involved in intracellular insulin degradation. The metalloendoprotease substrate analogues Z-Gly-Phe-NH_2 and Z-Gly-Leu-NH_2 also inhibited the intracellular processing of internalized insulin, but in addition also increased insulin binding (Table 1, Fig. 1) by increasing receptor affinity (Figs. 2 and 3). It is not clear whether this increase in affinity caused by the substrate analogues is due to their interaction with an endogenous metalloendoprotease since the more potent metalloendoprotease inhibitor, phosphoramidon did not increase surface binding (Table 1). Thus, the additional effects of these lipophilic agents on insulin receptor affinity may be a consequence of their interaction with lipid or protein membrane components, thereby altering the receptor microenvironment. However, we cannot rule out the possibility that the effects of these agents on insulin receptor affinity may be due to their interaction with an endogenous receptor associated metalloendoprotease by a mechanism different from that of phosphoramidon. Regardless of the specific mechanisms, it is significant that both the dipeptide metalloendoprotease substrate analogues and the inhibitor phosphoramidon blocked processing of internalized insulin. These agents should, therefore, prove useful in further characterization of the pathways of intracellular processing of insulin-receptor complexes.

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