MODULATION OF ^{125}I-INSULIN DEGRADATION BY RECEPTORS IN LIVER PLASMA MEMBRANES

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Received November 17,1976

SUMMARY. This study investigated the interactions of insulin with receptors in a purified mouse liver plasma membrane. Two processes were found when insulin was incubated with the liver plasma membranes at 30°C; 1) the binding of insulin to these membranes and 2) the degradation of insulin by one or more associated insulinases or proteases. These two activities were physically separated, analyzed, and it was found that bound insulin is a substrate for insulin degradation. In addition, the rate of degradation of free insulin in solution was found to be 1.1% degraded insulin/available insulin/min in a 30 minute period. This rate is compared to 25.3% degraded insulin/available insulin/min under similar conditions when insulin is bound to the plasma membranes. Thus, insulin binding was shown to aid in its degradation. These results suggest that a specific binding by at least one of the receptors for insulin orients the molecule such that its degradation is facilitated.

One mode of action of polypeptide hormones occurs on the surface of cells, with receptors in the cells' membrane (Sutherland and Rall, 1960; Pasten and Roth, 1966; Crofford, 1968; Cuatrecasas, 1969). Insulin action has been extensively analyzed, utilizing membranes from many cell types.

The binding of insulin to liver membranes has been shown to be time-dependent, capable of saturation, and most importantly, stereochemically specific (Desbuquois and Cuatrecasas, 1973; Neville, 1974; and Freychet, et al., 1971). The degradation of insulin by liver membranes has also been well characterized. Two systems have been proposed, one a simple proteolysis (Mirsky, 1949), the other a disulfide reduction (Tomizawa, 1959), for which Varandani (1973) has recently implicated the involvement of glutathione-insulin-transhydrogenase.

The relationship between binding and degradation of insulin by plasma membranes is presently controversial (Freychet, et al., 1972; Terris & Steiner, 1975). Whether the binding of insulin results in inactivation by degradation, or whether an insulinase degrades circulating insulin prior to its binding is unknown. This manuscript describes a time course for insulin binding and degradation to liver plasma membranes. These two processes have been physically separa-

ted and utilizing them independently it has been possible to demonstrate a facilitation of insulin degradation by receptor binding.

METHODS. Purified mouse liver plasma membranes were prepared by a modification of the method described by House and Weidemann (1970). Male Swiss Webster mice (35-40g), obtained from Simonsen Laboratories (Gilroy, California), were starved overnight, decapitated, bled, and their livers were excised. The tissue was minced and washed three times with medium H; 0.25M sucrose (Schwartz Mann), 5mM Tris (Sigma), pH 7.4 (4^{o}). The minced tissue was then homogenized in two volumes medium H at low speed in a Potter Elvehjem teflon-glass homogenizer (0.15-0.23mm clearance). The homogenate was filtered through four layers of cheesecloth, diluted to a final volume of 4-5ml/g tissue with medium H, and centrifuged twice at $10,000xg_{max}$ (4^{o}) for ten minutes to remove nuclei, mitochondria, and lysosomes. The supernatant was centrifuged for sixty minutes at $75,000xg_{max}$ (4^{o}) to pellet the crude plasma membranes. The membranes were then suspended by hand homogenization in a 10% Ficoll (Pharmacia), 5mM Tris, pH 7.4 (4^{o}) solution. Twenty milliliter aliquots of the suspended pellet were layered on a discontinuous Ficoll gradient (12 ml of 15%, 6 ml of 26%) and then centrifuged at $90,000xg_{ave}$ (4^{o}) for ninety minutes. The liver plasma membranes, isolated in the 10% Ficoll layer, were pooled, aliquoted, and stored at -70^{o}. All protein determinations were performed according to the method of Lowry (1951).

^{125}I-Insulin Binding and Degradation by Washed Membranes

Carrier-free ^{125}I-insulin (also referred to as monoiodoinsulin) was prepared using Elanco insulin (lot YZ0223AMX) by the Sodoyez, et al. (1975) modification of the method of Hamlin and Arquilla (1974). The buffer used in all procedures was 7mM sodium acetate, 7mM sodium barbital, 0.15M sodium chloride, pH 7.4 (4^{o}) with 1.5% human serum albumin (salt poor, Hyland). The albumin was dialyzed against 10^{-2}M EDTA prior to its addition to the buffer.

For the assays, thawed liver plasma membranes were diluted 1:3 with buffer and centrifuged at $27,000xg_{max}$ (4^{o}) for ten minutes. The supernatant (S1) was saved, and the pellet was washed in the same volume of buffer. This supernatant was discarded and the pellet (P1) was suspended in buffer to give a concentration of 6 mg P1 protein/ml.

^{125}I-insulin at a concentration of 10^{-11}M was allowed to incubate overnight at 4^{o} in buffer. For the assay, 3.8 ml of buffer and 1.0 ml of ^{125}I-insulin were added to three reaction pots at 30^{o} and incubated for fifteen minutes. To two vessels, 3 mg of P1 was added in 0.5 ml volume, while to the third vessel, an equal volume of buffer was added. The binding and degradation of the ^{125}I-insulin was measured by removing 0.2 ml aliquots from these vessels at times 0, .25, .50, 1, 2, 8, and 16 minutes. These aliquots were processed as described below.

After the 16 minute time point, 1.0 ml of either S1 or buffer was added to the appropriate reaction pot. Aliquots were taken at time .25, .50, 1, 2, 4, 8, 16, and 30 minutes after the addition and processed as described below.

Immediately following their removal, the aliquots were placed on ice and 0.5 ml of cold buffer was added. The aliquots were centrifuged at $27,000xg_{max}$ (4^{o}) for ten minutes and the pellets were washed once and resuspended in 0.5 ml of buffer. The combined supernatants and pellets were then precipitated with 10% trichloroacetic acid. After centrifugation as above, the precipitated pellets and their supernatants were counted on a Beckman Biogamma Counter (50% counting efficiency). The conversion of counts per minute to moles of ^{125}I-insulin was done using a calculated specific activity of 2.4×10^{o} cpm/picomole ^{125}I-insulin (assuming 100% iodonation).

RESULTS. In the presence of membrane, the monoiodoinsulin was increasingly bound through the 16 minute assay, reaching over 200 moles of monoiodoinsulin (10^{-17})/ mg protein (Figure 1). The degradation of the monoiodoinsulin was modest, amount-

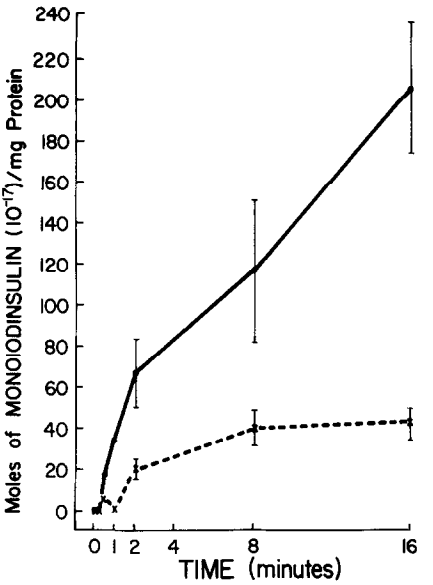

Figure 1: The Binding and Degradation of Monoiodoinsulin by Liver Plasma Membranes (P2)
Endogenous liver plasma membranes were washed with buffer to remove enzymatic activity. 3 mg of this membrane (P2) was incubated with 1 x 10^{-14} moles of monoiodoinsulin in a volume of 5.3 ml buffer at 30°C. Aliquots of 0.2 ml were removed and the binding (●——●) and degradation (x---x) were measured as described in the text. Background binding and degradation due to the presence of protein in the buffer was subtracted. The error bars represent standard deviation for the mean of 4 experiments.

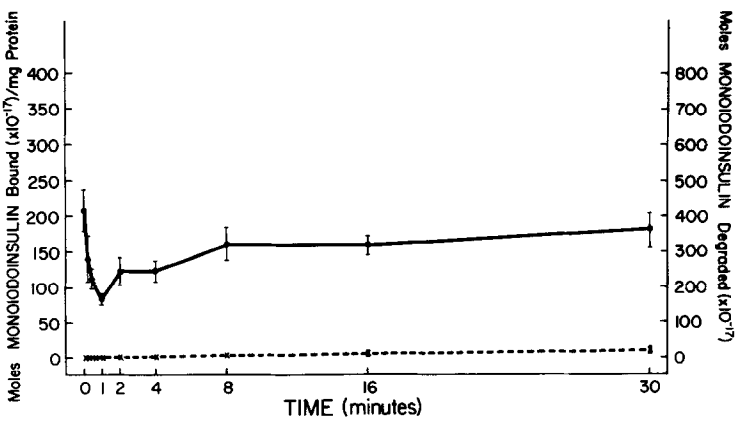

Figure 2: The Binding and Degradation of Monoiodoinsulin After the Addition of Buffer to Bound Monoiodoinsulin
After the 16 minute incubation of monoiodoinsulin with P2, 1.0 ml of buffer was added (at time 0 above). The binding (●——●) and degradation (x---x) of the monoiodoinsulin were measured, and background binding due to the presence of protein in the buffer was subtracted. The error bars represent standard deviations for the mean of 4 experiments.

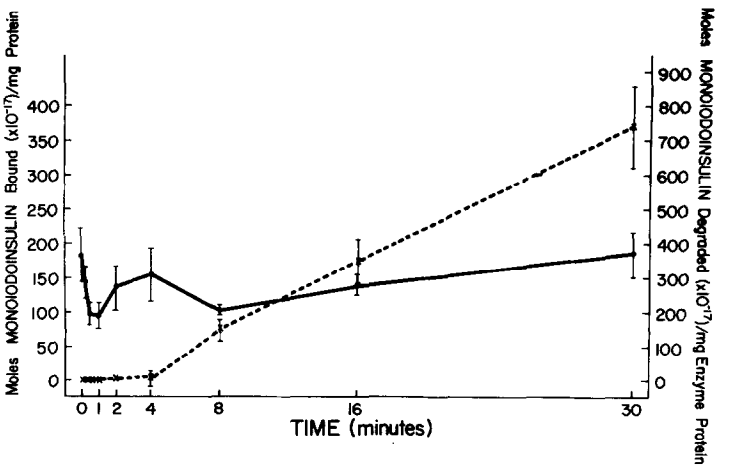

Figure 3: The Binding and Degradation of Monoiodoinsulin After the Addition of S1 to Bound Monoiodoinsulin
After the 16 minute incubation of monoiodoinsulin with P2, 1.0 (1.08 mg) of S1 was added (at time 0 above). The binding (●——●) and degradation (x---x) of the monoiodoinsulin were measured as described in the text. Background binding (from protein in the buffer) and degradation (from both the buffer and the membrane alone) were subtracted. The error bars represent standard deviations for the mean of 4 experiments.

ing to only 40 moles of monoiodoinsulin (10^{-17})/mg protein in the 16 minute assay. These findings indicated that the isolated mouse liver plasma membrane was capable of binding monoiodoinsulin with little subsequent degradation.

Degradation of Bound and Free Monoiodoinsulin - After 16 minutes of incubating monoiodoinsulin with and without liver membranes, the supernatant fraction (S1) of the membrane was added. In the control, buffer was added to the membrane insulin reaction mixture (Figure 2). The amount of monoiodoinsulin bound to the membrane decreased rapidly during the first minute following the addition of buffer. The binding of monoiodoinsulin was subsequently resumed and reached its initial value after 30 minutes. The amount of degraded monoiodoinsulin changed very little from that present before the addition of the buffer, indicating that the buffer was incapable of degrading the monoiodoinsulin.

When S1 was added to the membrane insulin reaction mixture, the amount of bound monoiodoinsulin decreased during the first minute in a similar fashion to the addition of buffer (Figure 3). The degradation of the monoiodoinsulin by the

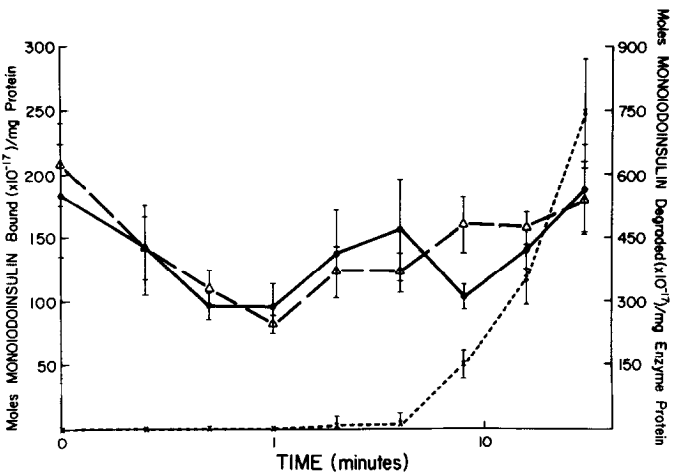

Figure 4: Comparison of the Binding of Monoiodoinsulin in the Presence and Absence of S1
This is a log plot of the binding of monoiodoinsulin in the presence (●——●) and absence (△——△) of S1. Also shown is the degradation of monoiodoinsulin by S1 (x---x). Error bars represent standard deviations for the mean of 4 experiments.

S1 fraction was rapid, starting after a 4 minute delay. By 30 minutes, the amount of degraded monoiodoinsulin was more than 700 moles (10^{-17})/mg enzyme protein. Of significance was the decrease of bound insulin noted at 8 minutes. This decreased binding at 8 minutes occurred coincident with the initiation of degradation and was absent when buffer was added (Figure 4).

When the supernatant fraction (S1) was added to the control buffer-monoiodoinsulin mixture, degradation of insulin proceeded at an attenuated rate compared to the rate of degradation observed in the presence of membrane (Figures 3 and 5). No 4 minute delay period for the initiation of degradation was observed in the absence of membrane. In addition to the degradation, it was noted that no binding occurred with S1. Therefore, the supernatant fraction was shown to contain no receptors capable of binding monoiodoinsulin.

Specific Degradation - In order to better quantify the differences observed in the rates of degradation of bound and free monoiodoinsulin, the data was converted to rates of specific degradation. This was done by dividing the amount of degra-

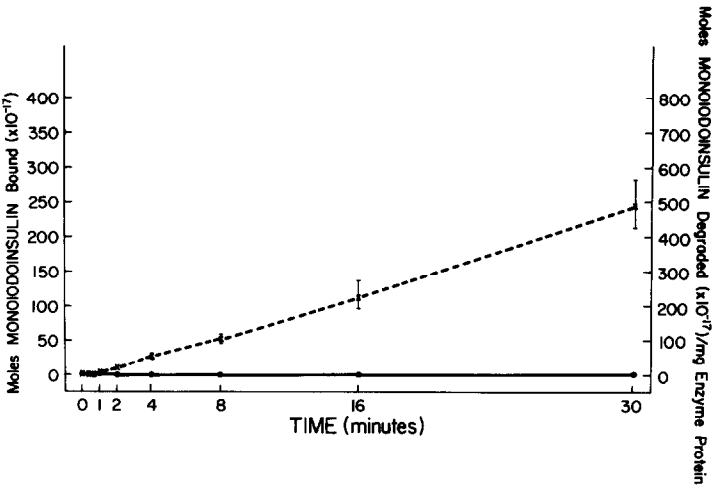

Figure 5: The Binding and Degradation of Monoiodoinsulin After the Addition of S1 to Free Monoiodoinsulin
After the 16 minute incubation of monoiodoinsulin with buffer, 1.0 ml (1.08 mg) of S1 was added (at time 0 above). The binding (●——●) and degradation (x---x) were measured. Background degradation and binding due to protein in the buffer was subtracted. The error bars represent standard deviations for the mean of 4 experiments.

ded monoiodoinsulin by the amount of monoiodoinsulin available to be degraded. By this calculation, the degradation of bound monoiodoinsulin was almost 25 times faster than that of the free monoiodoinsulin (25.3% degraded monoiodoinsulin/available monoiodoinsulin/min versus 1.1% degraded monoiodoinsulin/available monoiodoinsulin/min) (Figure 6). These two lines are statistically different ($P<0.01$) for each point after 2 minutes.

DISCUSSION. The work of Freychet, et al. (1972) has shown that insulin degradation by and binding to receptors in liver plasma membranes are independent processes. This conclusion is based upon data obtained from competition experiments with insulin analogues. In our studies, the two activities associated with the plasma membrane were physically separated and analyzed independently. In the presence of membrane, the binding of monoiodoinsulin decreased between 4 and 8 minutes following the addition of the enzyme (Figure 3). This decreased binding was not observed in the absence of enzyme (Figures 2 and 4). Furthermore, this decrease was concomitant with the rapid increase in monoiodoinsulin degradation

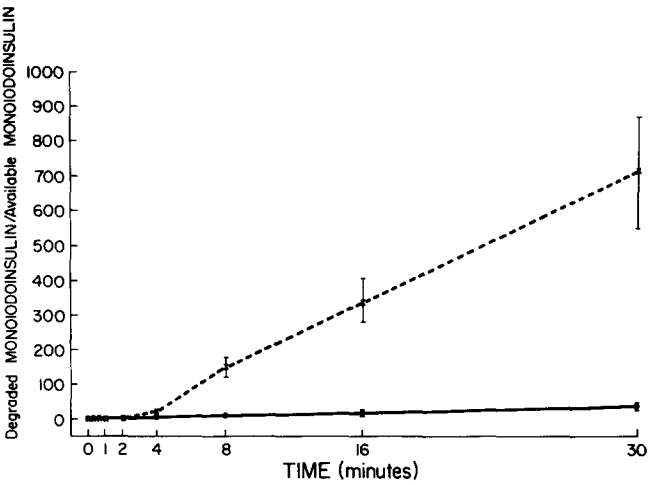

Figure 6: Specific Degradation of Monoiodoinsulin
The specific degradation was calculated for both the bound (x---x) and free (●——●) monoiodoinsulin as described in the text. The slopes of these lines were measured by linear regression methods as 25.3% degraded monoiodoinsulin/available monoiodoinsulin/min and 1.1% degraded monoiodoinsulin/available monoiodoinsulin/min for the degradation of bound and free monoiodoinsulin respectively. Correlation coefficients for both lines were .997. Error bars represent the standard deviations for the mean of 4 experiments.

(Figure 4). Therefore, we concluded that the enzyme is degrading monoiodoinsulin which is bound to the membrane. This result is in agreement with the recent observation of Terris and Steiner (1975) that bound insulin was a substrate for insulin degradation by intact hepatocytes.

Of significance was the demonstration of the facilitated degradation of mono iodoinsulin when it was bound to the membrane (Figure 6). This facilitation is of the order of 25 times in the presence of membrane than in its absence. In addition, this facilitation, coupled with the observation that bound insulin was a substrate for degradation, suggest that the stereochemically specific binding of insulin by membrane receptors orients the molecule in such a fashion that its degradation is facilitated. These studies support a theory that the modulation of insulins' action at tissues is controlled by enzymatic degradation.

In summary, the interaction of insulin with purified liver plasma membranes involves two processes, binding and degradation. These processes can be

physically separated and shown to occur independently. However, it was found that the prior binding of insulin to the plasma membrane facilitated its degradation. This facilitation is postulated to be a result of the specific orientation of the molecule by receptors in the plasma membrane.

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