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Quantitative studies of the rate of insulin internalization in isolated rat hepatocytes

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We studied internalization of ^{125}I-labelled insulin in isolated rat hepatocytes. Using the acidification technique, we were able to dissociate the ligand from its cell-surface receptors, and thus to separate internalized from surface-bound insulin. Because during the first 5 min of incubation of ^{125}I-labelled insulin with freshly isolated hepatocytes there is no loss of internalized label, the ratio of the amount of internalized ligand to the amount of cell-surface-bound ligand may serve as an index of insulin internalization. Within the first 10 min of insulin's interaction with hepatocytes, the plot of the above ratio as a function of time yields a straight line. The slope of this line is referred to as the endocytic rate constant (K_e) for insulin and denotes the probability with which the insulin–receptor complex is internalized in 1 min. At the insulin concentration of 0.295 ng/ml, the K_e is 0.049 min^{-1}. It is independent of insulin concentration until the latter exceeds 1 ng/ml. At the insulin concentration of 3.2 ng/ml, the K_e accelerates to 0.131 min^{-1}. With the K_e being the probability of insulin–receptor-complex internalization, 4.9% of occupied insulin receptors will be internalized in 1 min at an insulin concentration of 0.295 ng/ml, and 13.1% of occupied insulin receptors will be internalized in 1 min at 3.2 ng/ml. When the insulin concentration decreases from 3.2 to 0.3 ng/ml, the K_e decreases accordingly. The half-time of occupied receptor internalization is 15.4 min at the lower insulin concentration and 5.3 min at the higher insulin concentration.

Wiley & Cunningham (1982), using ^{125}I-labelled epidermal growth factor and cultured human fibroblasts, have proposed a method to quantify the endocytic rate constant, the probability for internalization of an occupied receptor in 1 min at 37°C. Using the acidification technique, they were able to separate internalized (In) from surface-bound (Sur) ligand. If at the time of measurement there is no degradation of the internalized ligand and the binding of ligand to cell surface approaches a constant, then the In/Sur ratio, plotted as a function of time, yields a straight line with a slope of K_e, the constant of the rate of endocytosis.

Assuming concomitant internalization of ligand and its receptor, we have applied the method proposed by Wiley & Cunningham (1982) in studying the endocytosis of insulin–receptor complexes in isolated rat hepatocytes. Having calculated the endocytic rate constant for insulin, we were able to quantify the number of insulin receptors entering the cell. The latter becomes extremely important in understanding the mechanism of insulin-induced receptor loss. The ability of ambient insulin concentration to regulate inversely the number of cell-surface receptors has been termed 'down-regulation' (Gavin *et al.*, 1974) and has been observed in isolated adipocytes (Marshall & Olefsky, 1980, 1981), cultured fibroblasts (Mott *et al.*, 1979; Baldwin *et al.*, 1981), human lymphocytes (Kosmakos & Roth, 1980; Kasuga *et al.*, 1981) and hepatocytes (Blackard *et al.*, 1978; Krupp & Lane, 1981). Nevertheless, the mechanism by which insulin induces loss of its own receptors remains unclear. It is now believed that insulin binding to its cell-surface receptors triggers endocytosis of the insulin–receptor complexes, and thus serves as a necessary initial step in inducing receptor loss (Olefsky *et al.*, 1982). Internalization of the insulin–receptor complexes may simply translocate insulin receptors from the cell surface into its interior, making them inaccessible to insulin. Alternatively, endo-

Abbreviations used: PhAsO, phenylarsine oxide; MEM, minimal essential medium.

cytosis of insulin receptors may accelerate the rate of their degradation. A third possibility is that the internalized insulin–receptor complexes diminish the rate of new receptor synthesis.

In this study we have attempted to assess the rate of insulin–receptor-complex internalization in isolated rat hepatocytes and relate it to ambient insulin concentration.

Materials and methods

Pig monocomponent insulin was generously supplied by Dr. Ronald Chance of Eli Lilly Co. ^{125}I-labelled insulin was purchased from Cambridge Medical Diagnostics; PhAsO was from Sigma; collagenase and hyaluronidase were from Worthington; and minimal essential medium (Earle's salts) was from Grand Island Biological Co.

Preparation of isolated hepatocytes

Hepatocytes were isolated from Sprague–Dawley adult rats (150–250g) by the method of Berry & Friend (1969), with modifications introduced by Terris & Steiner (1975), as previously described (Draznin *et al.*, 1981). The cells were resuspended in supplemented MEM containing 1% bovine serum albumin and 10mM-glucose. Cell viability in all experiments was above 95%, as judged by exclusion of 0.05% Trypan Blue.

Binding studies

Freshly isolated hepatocytes suspended in supplemented MEM were incubated with ^{125}I-labelled insulin in concentrations indicated in the Figure legends. At the different time intervals (1, 2, 3, 4, 5, 10, 15, 20, 25 and 30min), unbound insulin was separated from the cell pellet by centrifugation (600*g* for 2min) and the cell-associated radioactivity was determined. Cells were then incubated in 0.2M-acetic acid containing 0.5M-NaCl for 15min at 0°C. At this pH (2.5), surface-bound ligand dissociates from the cells, and the remaining cell-associated radioactivity represents internalized hormone (In). The difference between the amount of cell-associated radioactivity before and after acetic acid treatment yields the amount of cell-surface-bound ligand (Sur). The endocytic rate constant was calculated by the methods of Wiley & Cunningham (1982), where the constant (K_e) is the slope of the In/Sur ratio plotted as a function of time.

Results of binding experiments were corrected for non-specific binding (the amount of radioactivity attached to the cell pellet in the presence of 100μg of unlabelled insulin/ml) and expressed as a percentage of hormone bound to 10^6 cells.

The paired or unpaired Student's *t* test was used to compare mean values of different experiments as indicated. All experiments were conducted in duplicate in three to five independent experiments. All results were expressed as means $\pm$ S.E.M.

Results

In order to use the In/Sur ratio as the basis for calculating the endocytic rate constant, three conditions must be met (Wiley & Cunningham, 1982). The first condition is the complete separation of surface-bound ligand from the internalized ligand. The second conditions calls for a near-constant amount of cell-surface-bound ligand during the time of measurement. Lastly, there should be no loss of the internalized ligand during the time of measurement. Therefore we have tested the conditions in our experimental system to be sure that these criteria are met.

Although the acidification technique gained wide acceptance as a tool with which to remove surface-bound ligand, we have tested its ability to separate internalized insulin completely. Freshly isolated hepatocytes were incubated wtih ^{125}I-labelled insulin alone and with a combination of insulin and PhAsO (10μM) for 30min at 37°C. PhAsO has been previously shown to inhibit endocytosis irreversibly (Bradshaw & Rubin, 1980; Low *et al.*, 1981; Wiley & Cunningham, 1982). Hepatocytes were preincubated with PhAsO for 15min at 4°C before addition of insulin. In control experiments, hepatocytes were preincubated in MEM. The binding reaction was terminated at the indicated time intervals by centrifugation and the cell-associated radioactivity counted. The cell pellets were then incubated with acetic acid (pH2.5) for 15min at 4°C, washed once with 0.2M-acetic acid, centrifuged, and the cell pellet was counted for radioactivity present to determine the amount of internalized ligand. As shown in Fig. 1, insulin binding to isolated hepatocytes was rapid and identical in the presence and in the absence of PhAsO. When hepatocytes were incubated with insulin in the absence of PhAsO, acetic acid treatment removed only some of the ^{125}I-labelled insulin from the cells. When these hepatocytes were incubated with insulin in the presence of PhAsO, endocytosis was completely inhibited, and all ^{125}I-labelled insulin was removed from the cells by the acidification procedure. These results support previous observations that radioactivity remaining with the cells after the acidification procedure represents internalized ligand (Haigler *et al.*, 1980; Wiley & Cunningham, 1982; Sussman *et al.*, 1982; Olefsky & Kao, 1982; Caro *et al.*, 1982; Green & Olefsky, 1982). Acetic acid treatment does not remove internalized ligand, since further incubation of hepatocytes in acetic acid does not affect

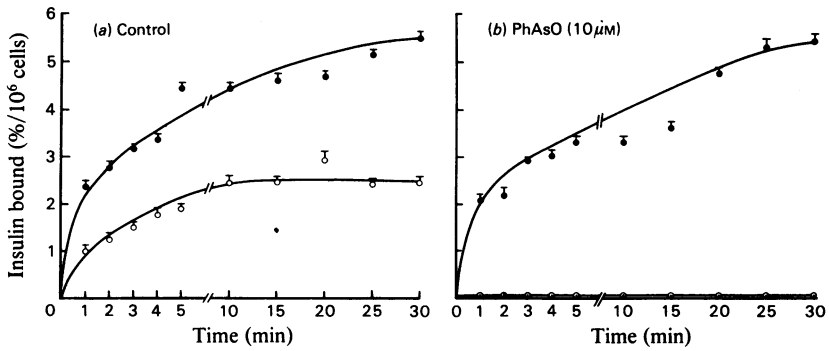

Fig. 1. *Insulin binding to isolated hepatocytes*
Hepatocytes were incubated with tracer concentration of insulin (0.295 ng/ml) in the absence (*a*) and in the presence (*b*) of PhAsO (10 μM) for 30 min at 37°C. At the indicated time intervals, hepatocytes were separated from the unbound ligand and the total cell-associated radioactivity (●) was determined. Hepatocytes were then treated with 0.2 M-acetic acid as described in the Materials and methods section, and the remaining radioactivity was counted. The latter represents internalized ligand (○). Results were corrected for non-specific binding and are plotted as means ± S.E.M. of three experiments, each conducted in duplicate.

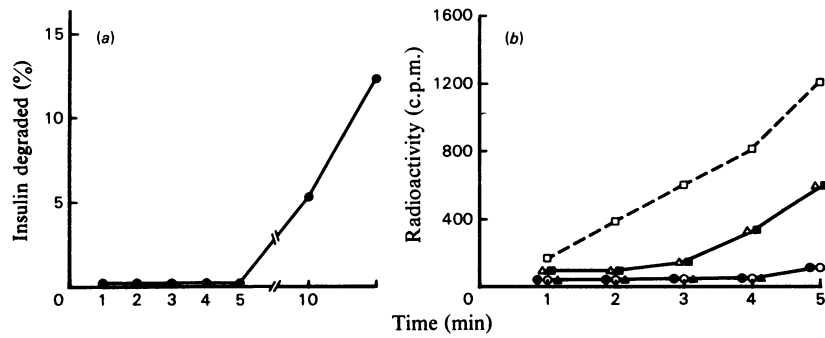

Fig. 2. *Release of insulin or its degradation products from hepatocytes*
(*a*) Appearance of trichloroacetic acid-soluble material in incubation media. Hepatocytes were incubated with insulin (0.3 ng/ml) at 37°C, and at indicated times samples of media were assayed for the appearance of acid-soluble material. Insulin degradation was calculated as follows:

$$\frac{\%\text{ degraded of incubated insulin}}{\%\text{ degraded of unexposed insulin}} \times 100$$

(*b*) Release of internalized ligand or its degradation products from isolated hepatocytes. The cells were incubated with ^{125}I-labelled insulin (0.3 ng/ml) for the indicated periods of time (●, 1 min; ○, 2 min, ▲, 3 min; △, 4 min; ■, 5 min; □, 10 min) at 37°C, washed twice with insulin-free MEM (at 0°C) and resuspended in insulin-free MEM at 37°C. The appearance of radioactivity in incubation media was followed for 5 min at 30 s intervals.

the amount of cell-associated radioactivity (results not shown). We conclude, therefore, that the acid stripping technique may be used to separate surface-bound from internalized ligand.

As shown in Fig. 1(*a*), insulin binding to isolated hepatocytes was rapid, and there was only a minimal increase (4–5%/min) in binding from the first until the fifth minute of incubation. Thus minute-to-minute changes in insulin binding during this time interval may be considered minimal and at 'near equilibrium', fulfilling the second requirement of the model used.

We then addressed the third important point in validating the approach used, namely that there be no loss of radioactive material from the cell interior during the period of measurement. If the internalized insulin were degraded, the appearance of trichloroacetic acid-soluble material in the incubation media would suggest the loss of internalized ligand through degradation. As shown in Fig. 2(*a*), there was no acid-soluble material in the incubation media within the first 5 min of incubation. If some molecules of intact insulin were to escape degradation and be released from the cell, this

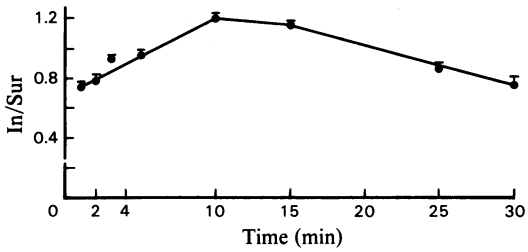

Fig. 3. *In/Sur ratio as a function of incubation time*
Hepatocytes were incubated with insulin (0.3 ng/ml) at 37°C for the indicated periods of time. Cell-associated radioactivity was determined before and after acetic acid treatment (see the Materials and methods section) to estimate the amounts of surface-bound (Sur) and internalized ligand (In).

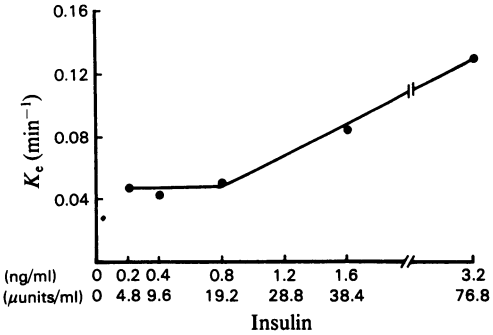

Fig. 4. *Endocytic rate constant (K_e) for insulin in isolated hepatocytes*
Hepatocytes were incubated with various concentrations of insulin for 10 min at 37°C. Amounts of surface-bound and internalized ligand were determined and plotted as a function of time. The slopes of these curves yielded the K_e. Results represent means of three experiments with each insulin concentration.

would be marked by the appearance of radioactive ligand in the incubation media. To check for this possibility, the cells with insulin bound to them (after 1, 2, 3, 4, 5 and 10 min of association) were washed twice in insulin-free MEM at 0°C in order to dissociate and remove cell-surface-bound ligand. The cells were then resuspended in warm (37°C) MEM, and the appearance of radioactive material in the medium was followed for 5 min at 30 s intervals. The cells associated with insulin for 5 min or less released no radioactive material in the media within the first 2–3 min after resuspension in warm MEM (Fig. 2*b*). These experiments suggest that there is no net loss of intracellular insulin within the first 5 min of incubation with ^{125}I-labelled insulin.

These data taken together indicate that our system meets all conditions necessary to calculate the endocytic rate constant by the model proposed by Wiley & Cunningham (1982). When the In/Sur ratio is plotted as a function of time (Fig. 3), a straight line is obtained during the first 10 min of incubation. The line does not pass through the origin, because of an extremely rapid binding reaction during the first 1 min of association of insulin with hepatocytes; 50% of maximal insulin binding was achieved at 1 min. This is twice as much as the amount of insulin bound to hepatocytes from the first until the fifth minute, or 10 times that observed at each subsequent minute of incubation. It is believed that rapidly increasing insulin binding during the first 1 min of incubation precludes the use of the In/Sur ratio for the assessment of the rate of insulin at this time. For the same reason we did not extend the In/Sur plot to the ordinate.

Insulin degradation, with subsequent release of degradation products from the cells, does not allow use of the curve after the initial 5 min for assessment of the endocytic rate constant. The slope of the straight line within the first 5 min is referred to as the constant of the rate of endocytosis of insulin. Fig. 4 demonstrates the dependence of the endocytic rate constant for insulin on the ambient insulin concentration; this constant is independent of insulin concentration until the latter exceeds 1 ng/ml (24 μunits/ml). The endocytic rate constant for insulin at the insulin concentration of 0.295 ng/ml (7 μunits/ml) was found to be 0.049 min^{-1}. Since the definition of the endocytic rate constant is the probability for internalization of the occupied insulin receptor, this number means that each occupied insulin receptor has a 4.9% chance of being internalized in 1 min. Another expression of this probability would be that 4.9% of the occupied insulin receptors would be internalized in 1 min. When insulin concentration is increased to 3.2 ng/ml (76.8 μunits/ml), the endocytic rate constant accelerates to 0.131 min^{-1}. This means that at this insulin concentration 13.1% of the occupied receptors would be internalized in 1 min.

The obvious conclusions from these experiments is that physiological increments in insulin concentration lead to the enhancement of insulin–receptor-complex internalization in isolated hepatocytes. The oscillations in ambient insulin concentration within the basal range (up to 24 μunits/ml) did not affect the rate of insulin–receptor-complex internalization.

We then approached the question of whether or not this acceleration of the rate of insulin–receptor-complex internalization is reversible, in other words, whether a return of insulin concentration to basal values will cause a decrease in the rate of internalization of the complex. To answer this

question, we incubated freshly isolated hepatocytes with 3.2ng of insulin/ml for 30min. After this incubation, insulin was removed and cells were placed into MEM with an insulin concentration of 0.295ng/ml. The endocytic rate constant of insulin–receptor-complex internalization was again measured. The endocytic rate constant decreased rapidly ($0.062\,min^{-1}$) after the decrease in ambient insulin concentration. These findings strongly suggest that the rate of insulin internalization follows physiological fluctuations in insulin concentrations.

Discussion

Our results clearly indicate that insulin regulates the rate of internalization of its own receptors. Interestingly, minor changes in ambient insulin concentrations within the basal range (up to 1ng/ml or 24μunits/ml) did not affect the rate of insulin–receptor-complex endocytosis. It is only when insulin concentration exceeds this value that the endocytic rate constant of internalization of the complex accelerates significantly. These observations are in keeping with the findings of other investigators that insulin enhances translocation of its receptor from the cell surface to intracellular space (Gavin *et al.*, 1974; Vigneri *et al.*, 1978; Green & Olefsky, 1982). Having calculated the endocytic rate constant, we were able to demonstrate that reversal of insulin concentration from high to low led to a decrease in the rate of insulin–receptor-complex internalization. These observations emphasize that short-term post-absorptive increases in insulin concentration, although enhancing the rate of insulin internalization, are completely and rapidly reversible.

Assuming that insulin is internalized via the receptor-mediated pathway, one can approach the question of whether ambient insulin concentration affects the rate of internalization of insulin receptors.

Isolated hepatocytes have 140000–150000 cell-surface insulin receptors per cell. Receptor occupancy at an insulin concentration of 0.295ng/ml is 1.84% (2650 receptors) and at an insulin concentration of 3.2ng/ml is 12.25% (17650 receptors). With the probability of receptor internalization (at a lower insulin concentration) being 4.9%, it means that 130 insulin receptors will be internalized in 1min with $t_{\frac{1}{2}}$ (half-life, $0.693/K_e$) for internalization of occupied receptors being 15.4min. For higher insulin concentrations (3.2ng/ml) the calculations are as follows: 17650 receptors are occupied; the probability of insulin–receptor internalization is 13.1%, which results in 2310 receptors being internalized in 1min ($t_{\frac{1}{2}}$ for internalization of occupied insulin receptors is 5.3min).

Results depicted in Fig. 1 require additional discussion. Inhibition of insulin internalization by PhAsO or dansylcadaverine (Draznin & Trowbridge, 1982) does not affect the amount of cell-surface-bound ligand. It seems that, at the time of binding equilibrium, new molecules of insulin bind to the cell-surface receptors only after already bound molecules either internalize or dissociate from the cells. Hence receptor occupancy at the given ambient insulin concentration is maintained independently of ligand internalization. On the other hand, because the internalized ligand adds to the total cell-associated radioactivity, one might expect the latter to be greater in control cells than in the cells treated with PhAsO, where internalization is inhibited.

Indeed, this phenomenon is seen during the initial 10–15min of incubation, when the rate of degradation of internalized ligand is minimal. Theoretically, the total cell-associated radioactivity in control cells should be greater than the total cell-associated radioactivity in cells treated with PhAsO by the amount of internalized insulin. In reality, we did not observe this magnitude of difference. At present, we can offer no explanation for this. Moreover, after the initial 10–15min of incubation, when the rate of degradation of internalized ligand is maximal and degraded insulin is not kept intracellularly, the minimal difference in the total cell-associated radioactivity is no longer seen. These experiments, however, were performed only to validate the acidification technique and do not impose any constraint on the subject of the paper.

There are certain assumptions that must be discussed and fully understood in order to interpret the data obtained. First, this model assumes that one molecule of labelled insulin is internalized along with one insulin receptor. If internalization of insulin is accompanied by an internalization of more than one insulin receptor, then all the calculations proposed above are invalid. We believe that, although cross-linking of insulin receptors may exist, only occupied insulin receptors undergo internalization. However, we cannot discard the possibility that a few unoccupied insulin receptors are internalized simply as a part of membrane-protein exchange. The second major assumption is that radioactive iodine, whether it is inside a cell or still on the cell surface, always represents radiolabelled ligand. The correction of all our data for non-specific binding presumably ensures that this assumption holds true. However, if occasional molecules of radioactive iodine that are not part of insulin enter these cells, the calculations of insulin–receptor turnover may be affected. A third assumption, that insulin enters its target cells only via receptor-mediated endocytosis, has been con-

firmed by numerous previous investigations (Terris & Steiner, 1975; Gorden *et al.*, 1978; Schlessinger et al., 1978; Goldfine et al., 1980; Olefsky et al., 1982; Gorden et al., 1982).

Notwithstanding these reservations, we believe that the data presented here are consistent with the current thinking about insulin–receptor-complex internalization. The fluctuations in insulin concentration within the physiological range elicit significant changes in the rate of internalization of both insulin and its receptors.

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