Internalization and degradation of a low affinity insulin ([Leu^{B24}]insulin) by rat adipocytes

Allan Green, Howard Tager*, Arthur Rubenstein*, Bruce Frank^{+} and Jerrold M. Olefsky

*University of Colorado Health Sciences Center, Department of Medicine, B151, 4200 East Ninth Avenue, Denver, CO 80262, * University of Chicago School of Medicine, Departments of Biochemistry and Medicine, 950 E. 59th Street, Chicago, IL 60637* and $^{+}$ *Lilly Research Laboratories, 307 East McCarty Street, Indianapolis, IN 46285, USA*

Received 24 May 1982

Insulin *Adipocytes* *Endocytosis* *Insulin receptors* *Degradation* *Chloroquine*

1. INTRODUCTION

Following initial binding to a specific cell-membrane receptor, insulin is taken up into the cell and degraded [1–4]. Very little is known of this endocytotic-intracellular degradation pathway. The structure of the insulin molecule is well understood, and one portion of the molecule which is important in binding to the receptor has been determined to be amino acids 22–26 of the B chain [5,6]. However, it is not known if this sequence is important in determining whether the molecule is internalized and processed intracellularly. In [7] it was shown that a Leu–Phe substitution in position 24 of the B chain results in an insulin molecule ([Leu^{B24}]-insulin) with a low binding affinity for the insulin receptor. Furthermore, the molecule antagonizes the biological action of normal insulin. Here, we show that the amino acid substitution does not alter the rate of internalization nor the rate or extent of degradation of receptor bound analog to low-M_r products. This demonstrates that the receptor binding region of the insulin molecule is not recognized in the degradative processes subsequent to the binding event.

2. EXPERIMENTAL

[Leu^{B24}]Insulin was prepared as in [7]. Adipocytes were isolated from rat epididymal fat pads by the method in [8] as in [9]. Insulin and [Leu^{B24}]insulin were iodinated by a modification [10] of the method in [11]. The techniques for measurement of [^{125}I]insulin binding, dissociation and degradation have been detailed in [12,13].

3. RESULTS AND DISCUSSION

The potency of [Leu^{B24}]insulin to inhibit binding of [^{125}I]insulin to adipocytes is shown in fig.1. It can be seen that the EC_{50} for inhibition of [^{125}I]insulin binding is ~70 ng/ml. This is compared to the EC_{50} for insulin's ability to inhibit [^{125}I]insulin binding,

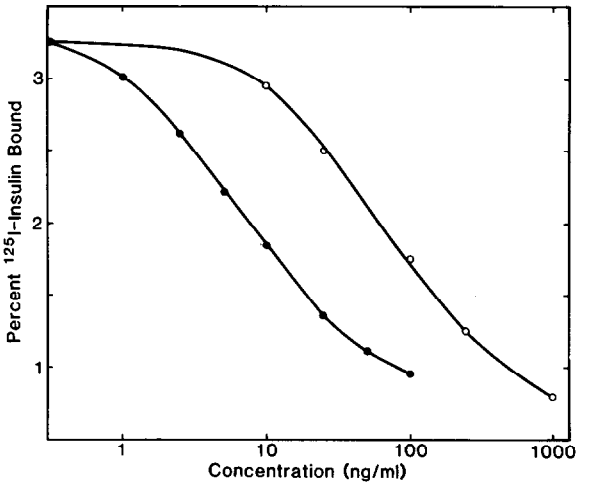

Fig.1. Effect of insulin and [Leu^{B24}]insulin on [^{125}I]insulin binding by adipocytes. Adipocytes (~2 × 10^5/ml) were incubated with [^{125}I]insulin (0.3 ng/ml) plus the indicated concentration of insulin (•) or [Leu^{B24}]insulin (○), for 90 min at 24°C. At the end of this period [^{125}I]-insulin binding to the cells was determined.

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75 © 1982 Federation of European Biochemical Societies

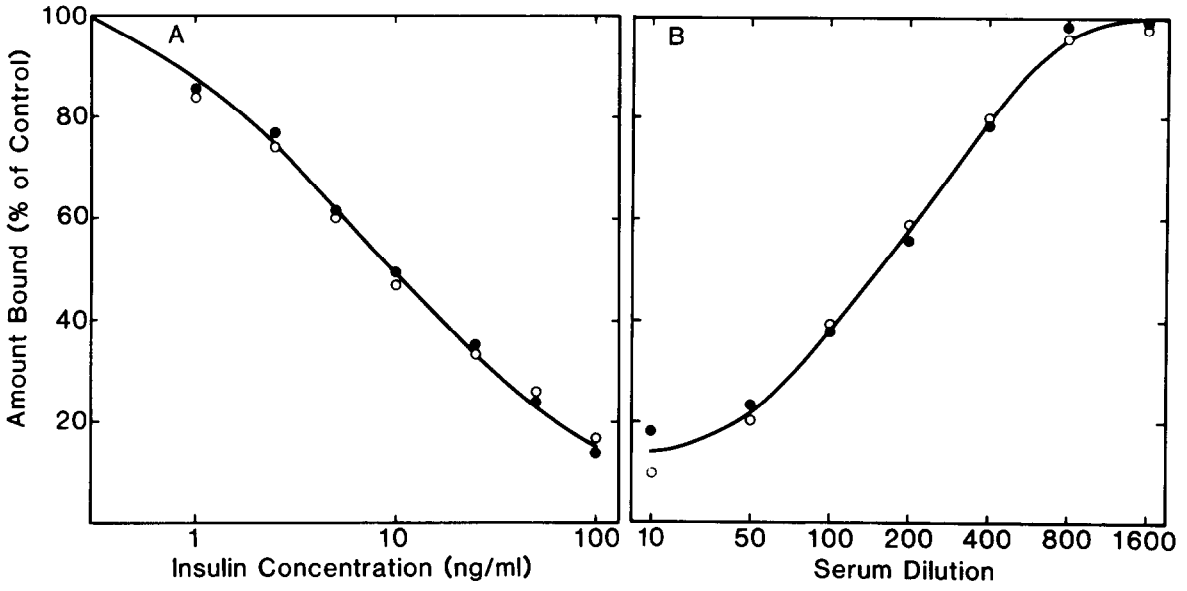

Fig.2. Effects of insulin and anti-insulin receptor antibodies on binding of [^{125}I]insulin and ^{125}I-labeled [Leu^{B24}]insulin to adipocytes. Adipocytes (~ 2 × 10^5) were incubated with 0.3 ng [^{125}I]insulin (•) or 1 ng ^{125}I-labeled [Leu^{B24}]insulin (○) in 1 ml buffer for 90 min at 24°C. At the end of this period the amount of [^{125}I]insulin or ^{125}I-labeled [Leu^{B24}]insulin bound was determined. (A) Effect of insulin, at the indicated concentrations, added together with the labeled hormones. (B) Effects of a serum containing anti-insulin receptor antibodies. The serum, at the indicated dilutions, was added to the cells 15 min before the labeled hormone.

which is ~ 10 ng/ml. By comparing these EC_{50}-values it can be seen that [Leu^{B24}]insulin has ~ 15% the binding affinity of insulin.

In order to ensure that ^{125}I-labeled [Leu^{B24}]-insulin binds only to the insulin receptors, the experiments described in fig.2 were performed. Fig.2A shows the effect of insulin on binding of [^{125}I]insulin and of ^{125}I-labeled [Leu^{B24}]insulin. It can be seen that insulin is equally potent at inhibiting binding of the two labeled molecules, strongly suggesting that they bind to the same receptors. A second line of evidence that ^{125}I-labeled [Leu^{B24}]insulin binds to the insulin receptor is shown in fig.2B. This experiment demonstrates that a serum containing anti-insulin receptor antibodies is equally potent at inhibiting binding of [^{125}I]insulin and of ^{125}I-labeled [Leu^{B24}]insulin to adipocytes. Taken together these results demonstrate that [Leu^{B24}]insulin binds with low affinity to the same population of insulin receptors as does native insulin.

Chloroquine can be used to assess the amount of insulin internalized by adipocytes [14]. This drug causes an increase in the amount of cell-associated [^{125}I]insulin when adipocytes are incubated with [^{125}I]insulin at 37°C. Chloroquine acts by inhibiting intracellular processing of insulin and subsequent release of degradation products [3,4]. Therefore the difference between cell-associated insulin in the presence and absence of chloroquine provides an estimate of the amount of hormone internalized [14]. Thus, we have compared the actions of the drug on the association of insulin and [Leu^{B24}]insulin to isolated adipocytes (fig.3). To compare the chloroquine effect independently of the difference in binding affinities of [^{125}I]insulin and [Leu^{B24}]insulin, it was necessary to use a higher concentration of [Leu^{B24}]insulin than of insulin, such that the amounts bound were approximately equal. This was achieved by using ^{125}I-labeled [Leu^{B24}]insulin at 1 ng/ml and [^{125}I]insulin at 0.2 ng/ml (fig.3). Under these conditions it can be seen that the chloroquine-mediated increase in cell-associated radioactive material is approximately equal for [Leu^{B24}]insulin and insulin. Since the chloroquine effect provides an estimate of internalization, this suggests that once bound,

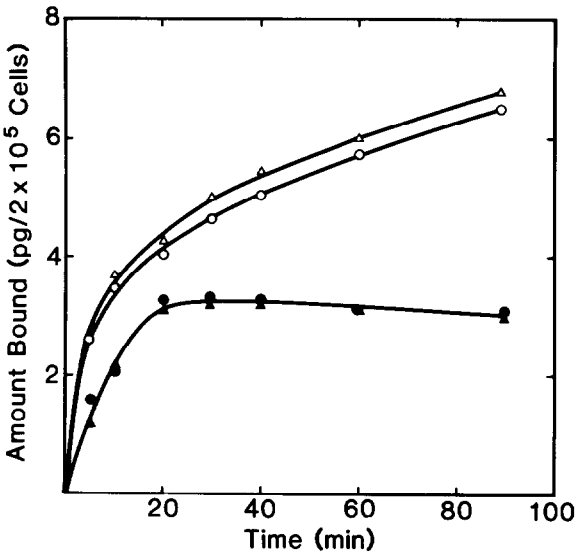

Fig.3. Effect of chloroquine on the association of [^{125}I]insulin and ^{125}I-labeled [Leu^{B24}]insulin. Adipocytes (~2× 10^5/ml) were incubated with 0.2 ng/ml [^{125}I]insulin (•,○) or 1 ng/ml ^{125}I-labeled [Leu^{B24}]insulin (▲,△) in the presence (open symbols) or absence (closed symbols) of 0.2 mM chloroquine, at 37°C. At the indicated times the amount of ^{125}I-labeled material bound to the cells was determined.

[Leu^{B24}]insulin is internalized by adipocytes at approximately the same rate as insulin.

When isolated adipocytes are incubated with [^{125}I]insulin the intracellular, or receptor-mediated, degradation forms only a portion of the total rate of insulin degradation [15]. Therefore, to study receptor-mediated degradation alone, the trichloroacetic acid solubility of material released from cells which have been pre-loaded with ^{125}I-labeled hormone is measured [15]. We have used this approach to compare receptor-mediated degradation of [Leu^{B24}]insulin and of insulin (fig.4). Since the apparent affinity constant (K_a) of insulin is related to the ratio of the association and dissociation rate constants, a potential problem was that the low affinity of [Leu^{B24}]insulin might be due to a fast dissociation rate, which would complicate interpretation of the results. However, [Leu^{B24}]insulin dissociates from adipocytes at about the same rate as insulin and its low affinity is probably due to a slow association rate constant (unpublished). Fig.4A shows the time-course of release of ^{125}I-labeled material from adipocytes pre-loaded with [^{125}I]insulin or ^{125}I-labeled [Leu^{B24}]insulin. It can be seen that the rate of release of ^{125}I-labeled material from cells loaded with ^{125}I-labeled [Leu^{B24}]insulin is the same as from cells loaded with [^{125}I]insulin. Furthermore, the trichloroacetic acid solubility of the material released from the 2 groups of cells was about equal at each time point (fig.B). This method (trichloroacetic acid solubility) assesses receptor

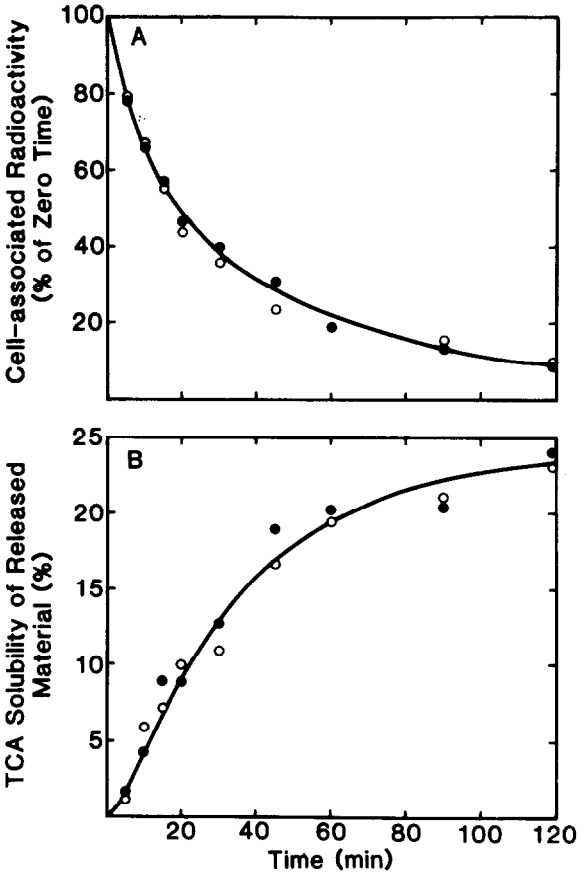

Fig.4. Receptor-mediated degradation of [^{125}I]insulin and ^{125}I-labeled [Leu^{B24}]insulin. Adipocytes (~4 × 10^5) were incubated with [^{125}I]insulin (0.4 ng/ml) or ^{125}I-labeled [Leu^{B24}]insulin (2 ng/ml) for 90 min at 24°C. Cells were then separated from medium, washed once in ice-cold buffer, re-suspended at 2 × 10^5 cells/ml and incubated at 24°C. At the indicated times the amount of ^{125}I-labeled material remaining bound to the cells (A) and the trichloroacetic acid solubility of the material released into the medium (B) were determined. (•) [^{125}I]insulin; (○) ^{125}I-labeled [Leu^{B24}]insulin.

mediated degradation to low-M_r products (mostly $^{125}I^-$ and iodotyrosine) and thus provides a measurement of the overall rate for processing the hormone to its final degradation products. However, although the overall rate of degradation of [Leu^{B24}]insulin is indistinguishable from that of the native hormone, recent experiments have identified differences among peptide intermediates of insulin and [Leu^{B24}]insulin metabolism by isolated hepatocytes (H.T. et al., unpublished).

These experiments demonstrate that [Leu^{B24}]insulin is internalized at the same rate as insulin, and that the overall rate of degradation of the hormone to low-M_r products is also comparable to that of the native hormone. Thus, an amino acid substitution in the receptor binding portion of the insulin molecule did not alter its overall rate of internalization or degradation, despite a marked decrease in binding affinity. Therefore the overall rates of receptor-mediated insulin internalization and degradation depend on the degree of hormone receptor occupancy, rather than on hormone structure. Although details of the intermediate proteolytic events in insulin degradation may involve elements of substrate specificity, our results show that the receptor binding region is not recognized in the degradative process subsequent to the binding event.

ACKNOWLEDGEMENTS

This work was supported by grants AM 18347, AM 13941, AM 20595, AM 25241 and AM 25242 from the National Institute of Arthritis, Metabolism and Digestive Disease.

REFERENCES

[1] Schlessinger, J., Schechter, Y., Cuatrecases, P., Willingham, M.C. and Pastan, I. (1978) Proc. Natl. Acad. Sci. USA 75, 5353–5357.
[2] Carpenter, J-L., Gorden, P., Freychet, P., LeCam, A. and Orci, L. (1979) J. Clin. Invest. 63, 1249–1261.
[3] Marshall, S. and Olefsky, J.M. (1979) J. Biol. Chem. 254, 10153–10160.
[4] Hammons, G.T. and Jarett, L. (1980) Diabetes 29, 475–486.
[5] Blundell, T., Dodson, G., Hodgkin, D. and Mercola, D. (1972) Adv. Protein Chem. 26, 280–294.
[6] Blundell, T. (1979) Trends Biochem. Sci. 4, 51–54.
[7] Tager, H., Thomas, N., Assoian, R., Rubenstein, A., Saekow, M., Olefsky, J. and Kaiser, E.T. (1980) Proc. Natl. Acad. Sci. USA 77, 3181–3185.
[8] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[9] De Pirro, R., Green, A., Yung-Chin Kao, M. and Olefsky, J.M. (1981) Diabetologia 21, 149–153.
[10] Freychet, P., Roth, J. and Neville, D.M. (1971) Biochem. Biophys. Res. Commun. 43, 400–408.
[11] Hunter, W.M. and Greenwood, F.C. (1962) Nature 194, 495–496.
[12] Marshall, S. and Olefsky, J.M. (1980) J. Clin. Invest. 66, 763–772.
[13] Olefsky, J.M., Green, A., Ciaraldi, T.P., Saekow, M., Rubenstein, A. and Tager, H. (1981) Biochemistry 20, 4488–4492.
[14] Marshall, S. and Olefsky, J.M. (1981) Diabetes 30, 746–753.
[15] Marshall, S. and Olefsky, J.M. (1980) Endocrinology 107, 1937–1945.