Insulin Binding to Isolated Human Adipocytes

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SUMMARY

In order to study the binding of insulin to insulin-sensitive human tissue, we isolated adipocytes from surgically obtained human fat tissue. Our data demonstrate that insulin readily and specifically binds to isolated adipocytes. The time course of this reaction indicates that steady state binding conditions occur at forty-five minutes, with a subsequent decline in binding later. There is no appreciable insulin or receptor degradation before forty-five minutes, suggesting a true equilibrium. After forty-five minutes the decline in binding can be accounted for by insulin plus receptor degradation. At 3 x 10^{-11} M 125-I-insulin and 2 x 10^5 cells per milliliter, 1.8 to 2.4 per cent of the 125-I-insulin was bound. The specificity of this binding reaction is demonstrated by the high concentrations of thyroid stimulating hormone and human growth hormone that are without effect on binding, while proinsulin and desalanine insulin inhibit binding in proportion to their biologic activity. On the other hand, binding can be readily inhibited by porcine insulin at physiologic concentrations, i.e. binding is 13 per cent inhibited at 1 ng. per milliliter and 50 per cent inhibited at 8.6 ng. per milliliter. The kinetic behavior of this reaction can be approximated by Scatchard analysis, which indicates 50,000 high affinity sites per cell with a dissociation constant (Kd) of 1.9 x 10^{-9} M per liter and 250,000 low affinity sites per cell with a Kd of 8 x 10^{-9} M per liter.

In conclusion, these studies demonstrate and characterize the binding of insulin to normal isolated human adipocytes; they indicate that the study of insulin-adipocyte binding is a possible means to gain further insight into the mechanism of altered response to insulin in human disease states. DIABETES *23*:565-71, July, 1974.

Since insulin exerts many, if not all, of its biologic effects without entering the cell interior,[1] it is thought that the first step in insulin action involves binding to a specific plasma membrane receptor. With animal tissues, insulin-receptor binding has been described using liver membranes,[2-6] adipocyte membranes,[7-9] thymocytes[10] and adipocytes.[11-14] Insulin binding has been less thoroughly characterized in humans, due to the difficulty in obtaining adequate tissue. Essentially all published data on insulin-receptor binding in man has been obtained utilizing the cultured or circulating lymphocyte as receptor source.[15-18] Although animal studies have indicated that the characteristics of insulin binding to lymphocytes are quite similar to those of other tissues,[9,19] lymphocytes are not a major site of insulin action. Consequently, the relationship of insulin-lymphocyte interaction to human physiology has been questioned.[16,17] Thus, it seems important to study insulin-receptor binding in a tissue which is an important site of insulin action. A brief report exists which indicates that human adipocytes can be used to study insulin binding, although no quantitative information is presented.[20] Therefore, in order to systematically characterize insulin-receptor binding in a tissue which is more related to insulin physiology in man, we decided to study adipocytes. The purpose of this report is to quantitatively characterize binding of insulin to the specific receptor located on isolated human fat cells.

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MATERIALS AND METHODS

Materials

Porcine monocomponent insulin, proinsulin, and desalanine insulin were generously supplied by Dr. Ronald Chance of the Eli Lilly Co. Human growth hormone (HGH) and thyroid stimulating hormone (TSH) were obtained from the N.I.H. hormone distribution committee. Sodium 125-I was purchased from Union Carbide, bovine serum albumin (Fraction V) from Armour and Co. and guinea pig anti-insulin antibody from Pentex Co.

Iodination of Insulin

I-125-insulin was prepared at specific activities of

150 to 250 μci. per microgram by the method of Freychet et al.[11]

Preparation of Isolated Fat Cells

Subcutaneous abdominal adipose tissue was surgically obtained from anesthetized adult subjects. All were nonobese, had normal carbohydrate metabolism and were consuming a normal diet until twelve to sixteen hours before surgery. Specimens were obtained at the beginning of surgery, as soon as the subcutaneous abdominal fat tissue was exposed. Isolated fat cells were prepared by shaking at 37° C. for sixty minutes in Krebs-Ringer bicarbonate containing crude collagenase (3 mg. per milliliter) and albumin (40 mg. per milliliter) according to the method of Rodbell.[21] Cells were then filtered through fine pore steel mesh, centrifuged at 400 rpm for four minutes and washed three times in a 25 mM Tris-1 per cent BSA buffer.[15]

Binding Studies

Unless otherwise indicated, isolated fat cells (1.0 to 4.0 x 10^5 cells per milliliter) were suspended in 1 ml. Tris-1 per cent BSA buffer, pH 7.5, and incubated with 125-I-insulin and unlabeled polypeptide hormones in siliconized 10 ml. Erlenmeyer flasks set in a 24° C. shaking water bath. The concentrations of 125-I-insulin and unlabeled hormone are given in the legends to the figures. The incubation was terminated by removing 200 λ aliquots from the cell suspension and rapidly centrifuging the cells in plastic microtubes to which 100 λ of dinonyl phthalate oil had already been added. According to the work of Gliemann,[14,22] dinonyl phthalate has a specific gravity intermediate between buffer and fat cells. Therefore, after centrifugation of the microtubes, three layers resulted: cells on top, oil in the middle and buffer on the bottom. The cells were then removed and their radioactivity was determined. All studies were done in triplicate.

RESULTS

Figure 1 depicts the progress of the binding reaction of 125-I-insulin to isolated human adipocytes with time. It can be seen that binding occurs rapidly at 24°, and reaches a peak at about thirty minutes (T max) which is maintained until at least forty-five minutes. Subsequent time points show a decline in the amount of bound radioactivity. At higher temperatures (37°) maximal binding is less, the steady state is much shorter, and there is a greater and faster decline in bound radioactivity. Consequently, in order

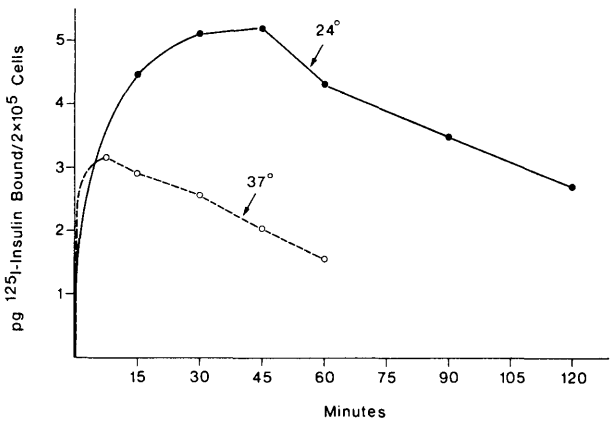

FIG. 1. Effects of time and temperature on the binding of 125-I-insulin to human adipocytes. Cells are incubated with 3.3 x 10^{-11} M 125-I-insulin at the indicated temperatures, and aliquots are removed at the indicated time points. The data represent the mean of three experiments and are corrected for nonspecific binding.

to assure more stable steady state conditions, all remaining binding studies were conducted at 24°.

The decline in binding which occurs at 24° is likely to be a result of receptor and/or insulin degradation. If degradation is also occurring before forty-five minutes, the presence of steady state binding conditions at thirty to forty-five minutes does not necessarily mean the binding reaction has reached equilibrium. It is possible that degradation of receptors and/or insulin, which would decrease binding, is occurring simultaneously with progressive increase in the binding reaction, and, therefore, the observed steady state could simply be a balance between these two processes. Consequently, in order to further characterize the decline in binding as well as the steady state seen in figure 1, we studied the progress of both receptor and insulin degradation with time.

Receptor degradation was measured by preincubation of adipocytes in the absence of insulin for the time periods specified in figure 2A. Following preincubation, 125-I-insulin was added, and the amount of binding was determined. This binding was compared to control tubes containing the same cell concentration and treated identically except for the lack of a preincubation period. Per cent degradation can be determined by the following formula:

$$1 - \frac{\text{amount bound by preincubated cells}}{\text{amount bound by control cells}} \times 100.$$

It can be seen from figure 2A that no significant degradation began until at least forty-five minutes.

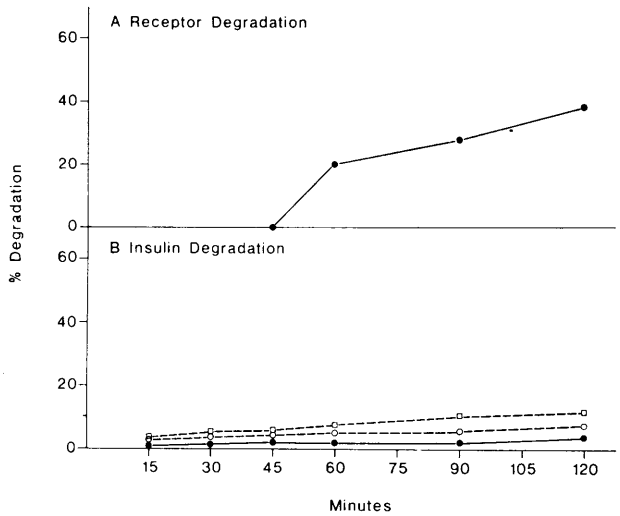

FIG. 2. Effect of time on receptor degradation (A) and on insulin degradation (B). Cells (2.2 x 10^5 per milliliter) are incubated with 3.3 x 10^{-11} M 125-I-insulin. The per cent of insulin degraded is determined by examining the ability of the 125-I-insulin remaining in the media to precipitate with TCA (●———●), to absorb to talc (0———0), and to precipitate with excess anti-insulin antibody (□———□). Data represent the mean of three experiments.

After this time point, receptor degradation increased in a somewhat linear fashion, reaching 28 per cent by ninety minutes and 38 per cent by 120 minutes.

Insulin degradation was determined by examining the ability of the 125-I-insulin remaining in the incubation media at the indicated time points to precipitate with 10 per cent TCA, to absorb to talc, or to bind to anti-insulin antibody. The per cent of the insulin which was intact according to the three methods was compared to that of insulin which was unexposed to adipocytes. Per cent degradation can be determined by the following formula:

$$1 - \frac{\%\ \text{intact of incubated insulin}}{\%\ \text{intact of unexposed insulin}} \times 100.$$

These data are shown in figure 2B. The degree of degradation is slight, but rises linearly with time; it should be noted that 94 to 98 per cent of the insulin was still intact after forty-five minutes of incubation. These results indicate that the decline in binding seen after forty-five minutes (figure 1) is associated with degradation of insulin and receptors, with receptor degradation predominating. Furthermore, very little degradation of receptors and insulin occurred before forty-five minutes, and measurements of insulin binding made at this time are likely to represent true equilibrium conditions. Because of the apparent equilibrium conditions at thirty to forty-five minutes, all remaining data were obtained at forty-five minutes of incubation.

The processes of degradation and decline in binding can be more accurately compared by quantifying the relationship between these two variables. This can be done by summing the per cent degradation of receptors and insulin shown in figures 2A and B, which should be equal to the per cent decline in binding due to degradation. The actual per cent decrease in binding with time can be calculated from the data in figure 1. Figure 3 compares the actual decline in binding calculated from figure 1 to the predicted decline based upon the data in figures 2A and B. The closeness of fit of these two lines suggests that the measured degradation of insulin and receptor sites adequately accounts for the loss of steady state conditions and subsequent fall-off of binding noted from the 24° time course data of figure 1.

In order to study the specificity of this binding reaction and to quantitate the competitive inhibitory effects of native insulin over a wide spectrum of concentrations, we examined the ability of insulin, insulin analogous, and a number of other polypeptide hormones to competitively inhibit the binding of 125-I-insulin to isolated fat cells. These experiments are summarized in figure 4. In these studies, 1.0 to 4.0 x 10^5 cells per milliliter were incubated with 125-I-insulin at a concentration of 3 x 10^{-11} M. Depending on cell concentration, maximal binding ranged from 0.9 to 4.8 per cent, and all data are expressed as a percentage of the amount of binding in the absence of unlabeled hormone. The binding is highly specific and cannot be inhibited by TSH or HGH. Furthermore, proinsulin and desalanine insu-

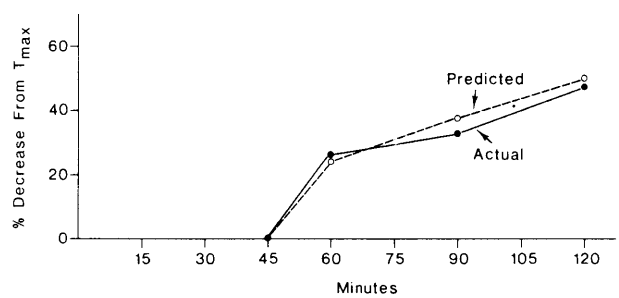

FIG. 3. Actual per cent decline in binding as calculated from the 24° time course data of figure 1 (●———●) as compared to the calculated decline in binding predicted by combining the degradation data of figures 2A and B (0---0).

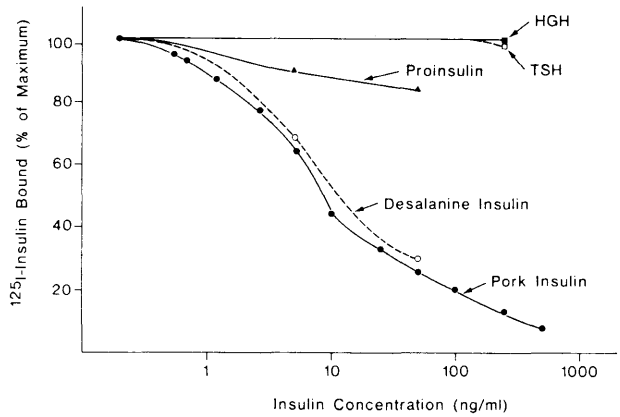

FIG. 4. The inhibiting effect of pork insulin (●——●), desalanine insulin (0---0), proinsulin (▲——▲), TSH (□---□) and HGH (■——■) on 125-I-insulin binding to fat cells. Incubations are carried out at 24° for forty-five minutes in the presence of 3.3 x 10^{-11} M 125-I-insulin. Unlabeled peptide concentrations are given on the horizontal axis. Data represent the mean of five experiments and are corrected for nonspecific binding.

lin inhibit binding proportional to their biologic activity. On the other hand, binding can be inhibited at small physiologic concentrations of native porcine insulin. At 1 ng. per milliliter (24 μU. per milliliter), 13 per cent of the binding is inhibited, and binding is 50 per cent inhibited (1/2 B max) at an insulin concentration of 8.6 ng. per milliliter. Twelve per cent of the bound radioactivity cannot be inhibited at an insulin concentration of 10^5 ng. per milliliter and is considered nonspecific binding. All data are corrected for nonspecific binding by subtracting the amount of 125-I-insulin bound at 10^5 ng. per milliliter insulin from the amount of 125-I-insulin bound at all other insulin concentrations.[2,12]

The relationship between cell concentration and binding is portrayed in figure 5. The amount bound is expressed as picograms 125-I-insulin, and it can be seen that binding is a linear function of cell concentration over a fivefold range. Since binding is a linear function of cell concentration (figure 5), the amount of insulin bound in the inhibition experiments can be normalized on the basis of cell concentration. When this is done, the data from these experiments can be combined, and the relationship between amount of insulin bound and insulin concentration can be analyzed. Figure 6 summarizes the amount of insulin bound per 2.0 x 10^5 cells per milliliter over the entire range of insulin concentrations. It can be seen that this relationship is curvilinear indicating saturation of receptor sites at higher insulin concentrations. From this graph one can calculate the number of insulin molecules bound per cell by converting nanograms of insulin to molecules of insulin and dividing by the cell concentrations. These calculations demonstrate that at insulin concentrations of 1 (24 μU. per milliliter), 10 and 100 ng. per milliliter 10,000; 48,000; and 200,000 molecules are bound per cell, respectively.

In an attempt to describe the kinetic behavior of hormone-receptor interactions, data similar to those presented in figure 6 have been analyzed by a variety of approaches.[14,16,23,24] All of these involve linearization of the data, and the most frequently used method is Scatchard analysis.[25] Using this form of linearization, the B max (maximal amount of insulin bound per 2.0 x 10^5 cells per milliliter) and the equilibrium constants (K_{diss} = dissociation constant, K_{aff} = affinity constant) can be approximated. When a Scatchard plot is constructed (figure 7), the data clearly do not describe one simple linear function. Similar findings with human lymphocytes[16] and rat liver membranes[26] have been interpreted as indicating at least two different orders of receptor sites. A similar interpretation of the data in figure 7 reveals a high affinity site with apparent equilibrium constants K_{diss} = 1.46 x 10^{-9} M per liter, K_{aff} 6.8 x 10^8 L/M; and a low affinity site with apparent equilibrium constants K_{diss} = 8.2 x 10^{-9} M per liter, K_{aff} = 1.2 x 10^8 L/M. Furthermore,

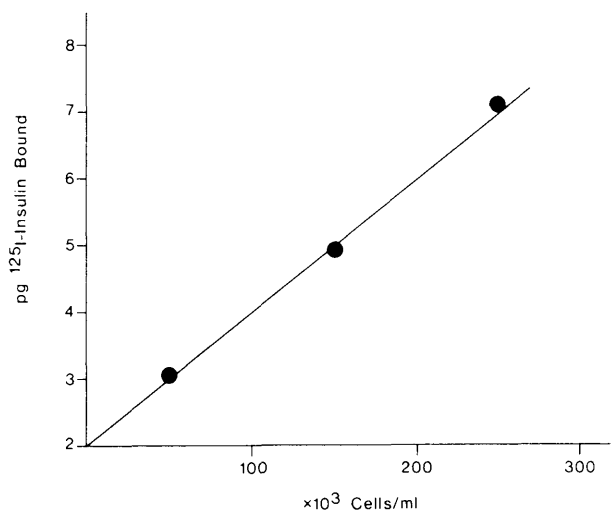

FIG. 5. Effect of cell concentration on 125-I-insulin binding to adipocytes. Incubations are performed in the presence of 10^{-10} M 125-I-insulin. Data represent the mean of three experiments and are corrected for nonspecific binding.

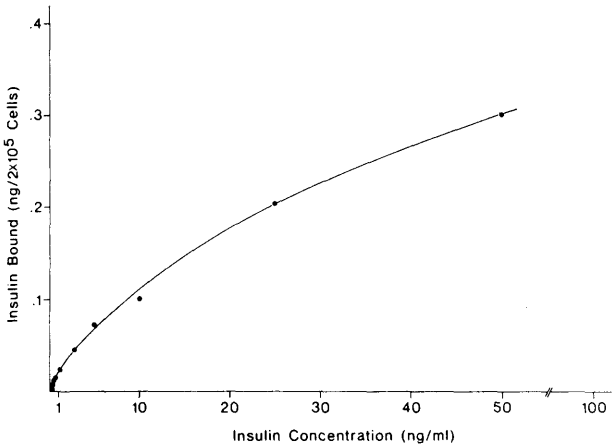

FIG. 6. Relationship between insulin concentration (horizontal axis) and amount of insulin bound in nanograms per 2 x 10^5 cells (vertical axis). At the lowest insulin concentration (0.2 ng. per milliliter), the per cent of insulin specifically bound per 2 x 10^5 cells ranged from 1.8 to 2.4 per cent.

one can estimate that there are 50,000 high affinity sites per cell and 250,000 low affinity sites per cell. For comparative purposes, the above constants were also calculated from a Lineweaver-Burke plot. In contrast, this plot was quite linear and suggested only one order of receptor site with apparent equilibrium constants $K_{diss} = 3.3 \times 10^{-9}$ M per liter, $K_{aff} = 3.0 \times 10^8$ L/M. The number of sites per cell that was estimated from this graph was 190,000.

DISCUSSION

These studies demonstrate that insulin readily binds to collagenase-prepared adipocytes obtained from normal anesthetized human subjects. This binding reaction reaches an apparent equilibrium, which subsequently falls off at later time points. We have demonstrated that degradation of both insulin and receptor sites—with the latter predominating —accounts for the decline in binding and the loss of steady state conditions. It should be mentioned that degradation of receptor sites is purely a descriptive term, and our data do not indicate what the actual event is, e.g. cell death, membrane turnover or membrane degradation. I-125-insulin binding is a linear function of cell concentration; the process is also temperature dependent, with decreased binding observed at higher temperatures. The amount of 125-I-insulin bound to human adipocytes can be competitively inhibited by concentrations of native insulin well within the physiologic range. Furthermore, HGH, TSH, proinsulin and desalanine insulin inhibit binding in proportion to their insulin-like biologic activity, as measured by the enhancement of glucose uptake. This demonstrates the specificity of the insulin receptor and its probable relationship to insulin action.

The binding of insulin to adipocytes is an equilibrium reaction which can be further characterized by knowledge of its kinetic behavior. Several methods have been suggested to analyze the kinetic behavior of hormone-receptor binding reactions;[14,16,23,24] we used the two most common approaches, i.e. Scatchard analysis and the Lineweaver-Burke method. It should be pointed out that none of these approaches has been adequately validated for hormone-receptor systems; all are dependent on a number of inherent (currently unproved) assumptions: data are obtained during equilibrium conditions, labeled and unlabeled hormones behave identically, one receptor molecule can bind only one insulin molecule, and no interactions

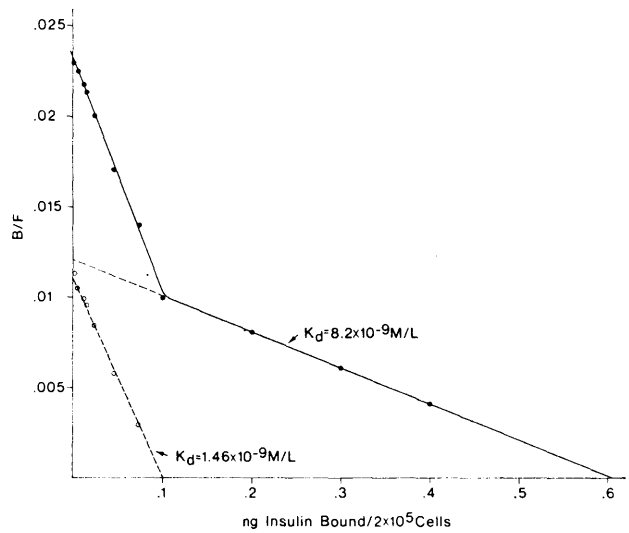

FIG. 7. Scatchard plot of the data in figure 6. The ratio of bound-to-free insulin is on the vertical axis and bound insulin is on the horizontal axis. The K_{diss} of the low affinity site is obtained from the slope of the least squares straight line drawn to fit the data obtained at insulin concentrations > 10 ng. per milliliter. The contribution of this line is then subtracted from the points obtained at lower insulin concentrations and a second least squares straight line is drawn to fit the resultant points. The K_{diss} of the high affinity site is obtained from the slope of this latter line. The number of receptor sites is calculated from the intercepts of these two lines on the horizontal axis.

between receptor sites exist. Using Scatchard analysis, two orders of receptor sites were detected: 50,000 high affinity sites per cell with an apparent K_{diss} of 1.5 x 10^{-9} M per liter and 250,000 low affinity sites per cell with an apparent K_{diss} of 8.2 x 10^{-9} M per liter. However, even though our data suggest that in the human fat cell system true equilibrium conditions probably do exist, because of the other qualifications outlined above, the values obtained from Scatchard plots must be cautiously interpreted. Using the Lineweaver-Burke method, we found one order of receptor site with 190,000 sites per cell having an apparent K_{diss} of 3.3 x 10^{-9} M per liter. However, as Bauliew et al.[23] have pointed out, it is difficult to detect a lower order site with this method when more than one order of binding site exists. It is evident that the K_{diss} obtained from the Lineweaver-Burke method and the high affinity K_{diss} obtained from Scatchard analysis are well within the physiologic insulin concentration range. Therefore, regardless of the method of analysis used or the exactness of the calculations, it is clear from these approximations that the binding reaction readily occurs at normal plasma insulin concentrations.

There are no similar published data on the human fat cell with which to compare these values. The only comparable data on insulin binding to human tissues has been obtained using the circulating lymphocyte.[15,16] With this approach, Gavin et al.[16] reported two orders of insulin receptor sites: a high affinity site with a K_{diss} of 0.5 x 10^{-9} M per liter and a low affinity site with a K_{diss} of 7.0 x 10^{-9} M per liter. We subsequently found essentially identical values using the lymphocyte.[27] Although the adipocyte has a much larger total number of sites per cell, the apparent equilibrium constants of human lymphocytes and human adipocytes are reasonably similar. However, these constants were all obtained using Scatchard analysis, and the assumptions inherent in this method were discussed above. For a more exact comparison, one can use the untransformed data to examine the insulin concentration which inhibits 50 per cent of the 125-I-insulin binding (1/2 B max). With the human adipocyte, this concentration is 8.6 ng. per milliliter. Using the lymphocyte, Gavin et al.[15] found this concentration to be about 10 ng. per milliliter, Archer et al.[17] reported 8.4 ng. per milliliter with the lymphocyte, and, also using the lymphocyte, we found a 1/2 B max of 9 ng. per milliliter.[27] This similarity of insulin binding to two entirely different human tissues suggests a general homogeneity of human insulin receptor behavior as has been demonstrated for the rat[10,14,28] and mouse.[19,26]

It is also of interest to compare the data we obtained with the human fat cell to data obtained using the rat adipocyte. For example, Kono et al.[13] calculated a K_{diss} of 6.6 x 10^{-9} M per liter, while Hammond et al.[8] found a predominant K_{diss} of 3.0 x 10^{-9} M per liter, and Gammeltoft et al.[14] reported a K_{diss} of 3.5 x 10^{-9} M per liter. Since these values are approximations, one might conclude that the dissociation constants for the insulin-receptor binding reaction in human and rat adipocytes are similar.

While it is clear that insulin readily binds to adipocytes at physiologic concentrations of insulin, it is important to compare this binding reaction to the biologic activity of insulin. The ability of native insulin to promote glucose uptake in human adipocytes has been reported to be maximal at an insulin concentration of about 1.4 x 10^{-9} M, with half maximal activity at 0.55 x 10^{-9} M per liter.[29,30] Using our binding data, it can be calculated that at these insulin concentrations only 23 and 14 per cent respectively of the available receptors are occupied. This suggests that only a small fraction of receptors need to be bound in order to give the full biologic response, or, not all receptors are identically linked to the subsequent biologic event. These findings are quite similar to those of Kono et al.,[13] who compared insulin binding and insulin-stimulated glucose uptake in rat adipocytes. Furthermore, based on theoretic considerations, Rodbard et al.[31] recently proposed similar concepts to explain the relationship between hormone binding and biologic activity.

In conclusion, these experiments indicate that the study of insulin binding to human adipocytes is possible, and it seems reasonable to believe that this approach will provide further insight into the mechanism of altered insulin responsiveness in human disease states.

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