BBA 76116

INSULIN BINDING TO PLASMA MEMBRANES

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(Received July 5th, 1972)

SUMMARY

Insulin binding to plasma membranes from rat epididymal fat pad, liver, brain and human red blood cell membranes was estimated by the displacement of [^{125}I]-insulin by native insulin. Membranes isolated from rat epididymal fat pad bound more insulin than rat liver plasma membranes. Rat brain and human red cell membranes bound essentially no insulin.

Glucose bound to fat pad membranes and this binding was decreased by insulin. The binding of labeled insulin was reduced in proportion to the relative concentration of labeled and unlabeled insulin. Maximum insulin binding required the presence of Na^+, K^+ and glucose. The binding of insulin was not altered by the insulin derivatives: sulfonated B chain, reduced–reoxidized insulin and oxidized B chain. The binding of insulin was inhibited in proportion to the concentration of added guinea pig anti-insulin serum. An increase in insulin binding was obtained following treatment of fat pad membranes with either phospholipase C or trypsin.

INTRODUCTION

It is well known that insulin has a direct effect of glucose metabolism in adipose tissue and muscle. Stadie *et al.*[1] demonstrated the binding of insulin to rat diaphragm muscle. Crofford[2] and Cuatrecasas[3] have shown that insulin exerts its action on the glucose metabolism of adipose tissue without entering the cell. Freychet *et al.*[4] have shown that monoiodoinsulin binds to fat cells and liver plasma membranes and is displaced by unlabeled insulin.

In the present study plasma membranes were isolated from epididymal fat pad, liver and brain of the rat and studies were carried out to determine which factors modify insulin binding to membranes. A preliminary report of this work has been presented (*Fed. Proc.*, 30 (1971) 1206).

EXPERIMENTAL PROCEDURE

Materials

^{125}I-labeled pork insulin (spec. act. 20–60 mCi/mg) was obtained from Abbott Laboratories. The purity was checked before use by the method of Ørskov[5]. Trypsin and phospholipase C were obtained from Sigma Co. Bovine serum albumin Fraction V

was obtained from Armour Co. Human insulin, reduced–reoxidized insulin, sulfonated B chain, zinc-free insulin and oxidized insulin were gifts from Dr Jerald Cantrell and Dr Robert Stroud. The anti-insulin serum at a dilution of 1:27000 precipitated 1 μunit of insulin. Other materials were analytical reagent grade and purchased from local suppliers.

Preparation of plasma membranes

Male Sprague–Dawley rats, weighing 150–175 g, maintained with free access to Purina Lab Chow and water, were fasted overnight and killed by decapitation. The tissues were excised and fat pad, brain and liver plasma membranes were prepared by the method of Ray[6], collecting the layer at the 1.16–1.18 *d* interface. Membranes were also prepared by the method of McKeel and Jarett[7] and no difference in control curves was obtained. Human red blood cells were obtained by centrifugation of heparinized blood. The cells were washed 2 times with 0.15 M NaCl and lysed by suspension in 10 vol. of water. The membranes were resuspended in water 2 times to insure complete lysing.

In the study to determine the effects of aging male Sprague–Dawley rats which weighed over 350 g and were over 1 year old were employed.

Incubation techniques

Isolated membranes were incubated in Krebs–Ringer bicarbonate buffer (pH 7.4) with 3.5 % albumin and 4 % glucose equilibrated with O_2–CO_2 (95:5, v/v). All incubations were performed in duplicate in siliconized glass flasks which were capped with rubber stoppers. The flasks contained 20 $\pm$ 2 μg (S.D.) of membrane protein (Lowry *et al.*[8]), at least 10000 cpm ^{125}I-labeled insulin and native insulin as indicated. A control curve was carried out during each experiment. Derivatives of insulin were added to follow competition with pork insulin or alone to follow inhibition of [^{125}I]insulin binding. All reactions were incubated for 1 h at 37 °C in a volume of 3 ml. At the end of the reaction period the contents of the flask were filtered over a Millipore filter (Type HAWP02500). The filters had been equilibrated in 0.05 M sodium phosphate buffer (pH 7.5), containing 5 % albumin, 0.1 % EDTA, and 0.25 % sodium ethylmercurithiosalicylate. The membranes were washed with 10 ml of this buffer to remove the free insulin. For each reaction a Millipore filter blank was included to determine [^{125}I]insulin bound non-specifically to the filter. Glucose binding to isolated fat membranes was determined as follows: Membranes were isolated from epididymal fat pad and reacted in Krebs–Ringer bicarbonate containing 3.5 % albumin. 30 μmoles of [U-^{14}C]glucose was added to each reaction in the presence of increasing amounts of insulin. After a 1-h incubation the reaction mixture was centrifuged at 1500 $\times$ *g* for 10 min. The pellet was suspended in 3 ml of 0.001 M $NaHCO_3$ and layered over a sucrose gradient which contained 3 ml each of 1.20, 1.18 and 1.16 dense sucrose. This was centrifuged at 90000 $\times$ *g* for 75 min. The membrane layer was collected, dissolved in 0.5 M NaOH, added to 20 ml Bray scintillation fluid[9] and counted in a Nuclear Chicago Mark II scintillation counter.

RESULTS

When [^{125}I]insulin was incubated with epididymal fat and a homogenate prepared and fractionated by centrifugation essentially all of the bound radioactivity was found in the membrane fraction

Insulin binding to fat pad membranes increased linearly up to 2 mg/ml (Fig. 1). A further increase in binding occurred at higher concentrations of insulin, but the rate of additional binding decreased markedly.

The binding of insulin as a function of the age of the animal was studied with membranes prepared from young and old rats. Membranes prepared from fat pads of older rats bound half as much insulin as those from younger rats (Fig. 1).

Guinea pig anti-insulin serum inhibited insulin binding in proportion to the amount added In the initial experiments the amount of anti-serum necessary to bind more than half of the added insulin was determined. The effect of the addition of this amount of antibody on the binding of insulin is also shown in Fig. 1.

Maximum insulin binding was obtained in the presence of both Na^+ and K^+. The effects of different ions on insulin binding was investigated by preparing Krebs–Ringer bicarbonate buffers each of which did not contain one of the ions: Na^+, K^+, Ca^{2+} or Mg^{2+}. Other buffers were prepared which contained Na^+ and/or K^+ Insulin binding to epididymal fat pad membranes was measured in these buffers and in

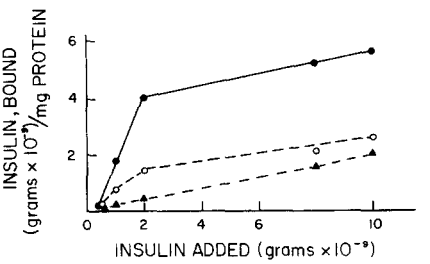

Fig 1 Insulin binding to fat membranes Membranes were isolated and reacted as described. ●—●, binding of insulin to fat membranes prepared from young, control rats, ○---○, insulin binding to fat membranes prepared from rats over 1 year old and weighing over 350 g; ▲–·–▲, insulin binding to fat membranes from control rats in the presence of anti-insulin guinea pig serum, which was present in each sample at one half the concentration required to precipitate the insulin present

TABLE I

BUFFER COMPOSITION, AND pH EFFECT ON INSULIN BINDING TO FAT MEMBRANES

KRB, Krebs–Ringer bicarbonate buffer which has been gassed for 10 min at 37 °C The below reactions below were incubated in the presence of [^{125}I]insulin and 50 μunits/ml of pork insulin A series of Krebs–Ringer bicarbonate buffers were prepared in which the indicated ions were omitted or glucose was omitted or the pH adjusted with NaOH or HCl. The flasks were incubated and the contents filtered as described These differences are highly significant ($P<0.001$) when matched with their controls and analysed by the paired t test ($n = 4$)

Buffer	*μunits insulin bound/mg protein*
KRB complete with 3% albumin and 4% glucose	108
Ca^{2+} and Mg^{2+} omitted from KRB with 3% albumin and 4% glucose	164
Ca^{2+}, Mg^{2+} and K^+ omitted from KRB with 3% albumin and 4% glucose	12 9
Ca^{2+}, Mg^{2+} and Na^+ omitted from KRB with 3% albumin and 4% glucose	56 7
Glucose omitted from KRB with 3% albumin	35 2
KRB with 3% albumin, 4% glucose (pH 7 1)	20 6
KRB with 3% albumin, 4% glucose (pH 7 2)	12 5
KRB with 3% albumin, 4% glucose (pH 7.5)	50 0
KRB with 3% albumin, 4% glucose (pH 7 8)	13.6

glycylglycine buffer and compared to binding in a complete Krebs–Ringer buffer. The results obtained are shown in Table I and indicate that both Na^+ and K^+ are required but Ca^{2+} and Mg^{2+} are not required. Indeed the omission of both Ca^{2+} and Mg^{2+} resulted in increased binding. The effect of glucose on insulin binding was determined by employing Krebs–Ringer bicarbonate buffers whose concentration of glucose varied from 0 to 4 %. The binding of insulin to fat plasma membranes was significantly modified by the presence or absence of glucose as shown in Table I.

Since glucose was determined to be a necessary factor for insulin binding, the binding of glucose to isolated fat pad membranes was determined. In the absence of insulin 12.5 μmoles of glucose were bound per mg membrane protein. Glucose binding was decreased in the presence of added insulin as shown in Fig. 2.

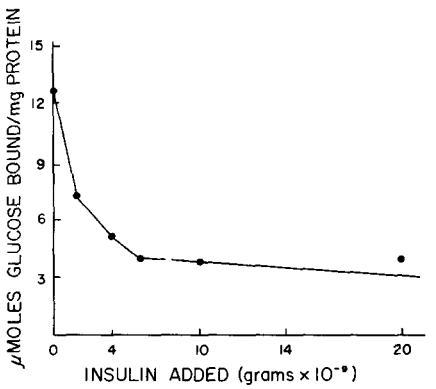

Fig 2. Glucose binding to fat membranes prepared from control rats Membranes were reacted in the presence of [U-^{14}C]glucose and increasing amounts of pork insulin as shown

The effect of pH on insulin binding was determined using Krebs–Ringer bicarbonate buffers containing 3.5 % albumin and 4 % glucose. The buffers were gassed and the pH adjusted with HCl or NaOH. The optimal pH for insulin binding to epididymal fat pad membranes was 7.5 (Table I).

Competition studies were performed using the reduced B chain of insulin, human insulin, zinc-free insulin and pork insulin. The displacement of [^{125}I]insulin from membranes was followed. Human insulin and zinc-free insulin displaced bound ^{125}I-labeled pork insulin similar to pork insulin: however, sulfonated B chain did not displace bound [^{125}I]insulin (Fig. 3). To determine if sulfonated B chain, reduced–reoxidized or the oxidized B chain of insulin inhibited the displacement of bound ^{125}I-labeled pork insulin by pure pork insulin these derivatives were added in equal quantities to pure pork insulin and the displacement of [^{125}I]insulin was followed (Fig. 4). These experiments indicated that neither of these insulin derivatives inhibited the displacement of bound [^{125}I]insulin by pure pork insulin. Reduced B chain and cysteine markedly increased insulin binding. A small increase was obtained in the presence of dithiothreitol (Fig. 5).

The binding of insulin to membranes isolated from rat fat, liver, brain and human red blood cells was examined. [^{125}I]Insulin bound greatest to fat pad membranes, less to liver membranes and the binding to brain and red blood cell membranes was essentially zero (Fig. 6).

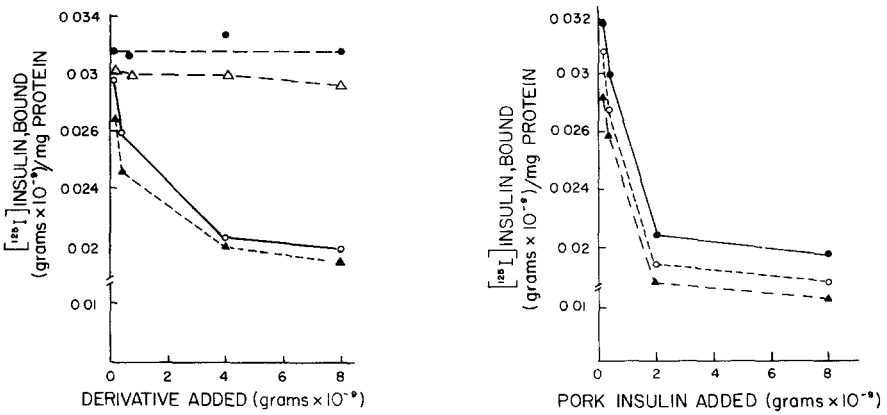

Fig 3 Displacement of bound [^{125}I]insulin from fat membranes by derivatives of insulin ●---●, displacement by sulfonated B chain, ○—○, displacement by pork insulin; ▲--▲, displacement by human insulin, △-·-△, displacement by reduced–reoxidized insulin

Fig 4. Competition between [^{125}I]insulin, pure pork insulin, and derivatives of insulin ●—●, competition between ^{125}I-labeled pork insulin and pure pork insulin, ○---○, the effect of the presence of sulfonated B chain on pork insulin displacement of [^{125}I]insulin, ▲-·-▲, the effect of the presence of oxidized B chain on pure pork insulin displacement of [^{125}I]insulin

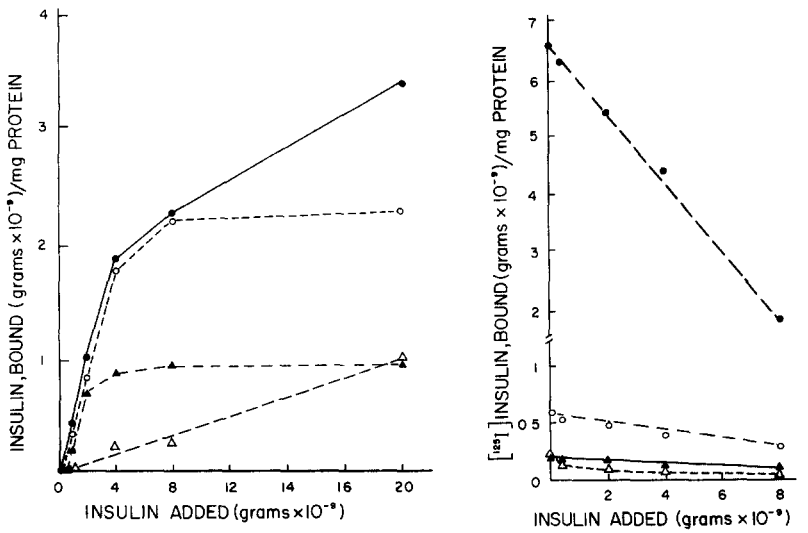

Fig 5. The effect of sulfhydryl compounds on insulin binding to fat membranes ●—●, binding of insulin in the presence of reduced B chain (the concentration of B chain on a g basis was the same as that of insulin at each point), ○---○, binding of insulin in the presence of 5 μg of cysteine; ▲-·-▲, control system; △--△, binding in the presence of 5 μg of dithiothreitol

Fig 6 Insulin binding to plasma membranes prepared from fat, liver, brain, and red blood cells. ●--●, binding to fat membranes ○-·-○, binding to liver membranes ▲—▲, binding to brain membranes △--△, binding to red cell membranes

Fat pad membranes were treated with 1 mg of trypsin or phospholipase C and re-isolated on a sucrose gradient. Insulin binding was determined using re-isolated membranes. Trypsin treatment slightly enhanced the binding, whereas phospholipase C greatly enhanced the binding (Table II).

TABLE II

INSULIN BINDING TO MEMBRANES AFTER TREATMENT WITH PHOSPHOLIPASE C OR TRYPSIN

Plasma membranes were isolated from rat epididymal fat pad and incubated at pH 7 5 with 1 mg of trypsin or phospholipase C for 30 min The membranes were washed by resuspension in 0 001 M $NaHCO_3$ and re-isolated on the sucrose gradient The layer at 1 14 *d* was suspended in $NaHCO_3$ and incubated in the presence of 100 μunits of insulin

Treatment	*ng insulin-bound per mg membrane protein*
None	7 21
1 mg trypsin	11 3
1 mg phospholipase C	21 1

TABLE III

INSULIN BINDING CONSTANTS

K was calculated according to Cuatrecasas[9] K' is the ratio of free to bound insulin These differences are highly significant ($P<0$ 001) when matched with their controls and analysed by the paired t test ($n = 4$).

System	K (pM)	K' (pM)
Complete	1 28	1 21
Glycylglycine buffer	0 634	1 67
K^+ free	18 35	39 41
Na^+ free	7 87	3 67
Older rats	1 79	6 93
Omit glucose	2 30	1 85
Liver	9 21	12 14

Binding constants were determined using both apparent (R) values and actual (R) values. The binding constant for control experiments was $1.28 \cdot 10^{-12}$ M. Calculating the constants in this way makes the assumption that the value of (R) remains constant, however, under different conditions it is possible that the receptor sites might be masked. For this reason we have defined a binding constant K' which is the ratio of bound to free insulin.

Using this equation binding constants K' are always higher than control values when less insulin was bound. Table III shows the values obtained under different conditions. The control values were relatively constant, the coefficient of variation being 9.1 % ($n = 20$).

DISCUSSION

Crofford[2] has shown that insulin is bound by intact epididymal fat cells in proportion to its effect on glucose oxidation, and Cuatrecasas[3] has shown that insulin bound to Sepharose which does not penetrate the cell causes an uptake of glucose by fat cells. Both findings indicate that insulin interacts with the plasma membranes to produce its effect.

Cuatrecasas and co-workers[10,11] has reported a binding constant for liver membranes of $6.7 \cdot 10^{-11}$ M and for fat cells $8.1 \cdot 10^{-11}$ M. Employing Krebs–Ringer buffer containing glucose we have obtained constants of $9.21 \cdot 10^{-12}$ and $1.28 \cdot 10^{-12}$ M for liver and fat membranes, respectively. It is apparent that glucose significantly increases insulin binding to these membranes and that fat pad membranes have a greater affinity for insulin than liver membranes.

In addition Cuatrecasas[11] has reported an increase in binding of insulin to liver membranes following treatment with phospholipase C. Our studies confirm this finding and show a similar effect with fat pad membranes. Increased binding following trypsin treatment was also observed. The treatment of membrane with such agents appears to make more receptor sites available to insulin.

Stadie and Zapp[12] have reported that insulin did not stimulate glycogen synthesis in the rat diaphragm when K^+ was the only cation present. In addition Hagen *et al.*[13] have shown that if Na^+ and K^+ are omitted from Krebs–Ringer bicarbonate buffer less glucose is oxidized by adipose tissue to CO_2 in the presence of insulin. We have shown that maximal insulin binding is dependent on added Na^+ and K^+ indicating that the decreased effect of insulin observed by the above authors is due at least in part to decreased binding of insulin to its receptor site.

Glucose binds to the isolated membranes, however, added insulin decreases glucose binding (Fig. 2) A decrease in glucose binding to the inner aspect of the membrane would account for this observation and the effect of insulin to increase glucose transport, however, the present design does not allow us to localize the binding site The increased binding in the absence of Ca^{2+} and Mg^{2+} is unexplained. Addition of Ca^{2+} and Mg^{2+} to glycylglycine did not modify the results obtained. The low solubility of Ca^{2+} and Mg^{2+} in bicarbonate buffer limited our experimental design. The results suggest that Ca^{2+} and Mg^{2+} interfere with the effect of Na^+ and K^+.

In addition Hagen *et al.*[13] have shown that fat pads isolated from rats weighing over 250 g gave less of a response to insulin than those rats weighing 150 g Plasma membranes isolated from rats which weighed over 350 g bound 50 % of the insulin bound by membranes from young rats which weighed less than 200 g. This finding suggests that aging modifies the insulin receptor or there are fewer receptors in older animals. Since we have shown that phospholipase or trypsin treatment increases insulin binding the latter possibility is indicated.

These studies suggest that the conformation of the membrane is of particular importance for insulin binding. Glucose aging, pH and enzyme treatment modify the insulin receptors or change their availability for insulin binding. Changes in a metabolic parameter resulting from *in vivo* or *in vitro* changes in the extracellular fluid may not simply reflect a modification of the intracellular effect of insulin but may be the result of increased or decreased interaction of insulin with its receptor site.

ACKNOWLEDGMENTS

This study was supported in part by N.I.H. Grant AM 15235 and by the VA Birmingham, Alabama.

The authors would like to thank Mrs B. Poole and E. M. Pacheco for their excellent technical and secretarial assistance.

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