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Insulin-Receptor Interactions. Possible Involvement of Metals

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Addition of Zn^{2+} or Cu^{2+} ions to plasma membrane preparations or to purified insulin receptors from rat liver resulted in an increase of specific insulin binding; no effect was observed with the addition of Fe^{3+}, Ca^{2+} or Na^{+}. Dialysis of membrane preparations, or of purified receptors, against chelating agents such as zincon (2-carboxy-2'-hydroxy-5'-sulfoformazyl-benzene) or 1,10-phenantroline resulted in a decrease in specific binding of insulin. With the readdition of Zn^{2+} or Cu^{2+} to the medium an increase in specific binding was observed, and values much higher than those of the original preparations were obtained; the addition of Ca^{2+}, Fe^{3+} or Na^{+} to dialyzed preparations did not cause any effect on the specific binding. Dialysis of purified receptors against chelating agents resulted in a decrease in the content of Zn^{2+} and Cu^{2+}. Zincon has been found to be a competitive inhibitor of insulin interfering with the specific binding to the receptor, and noncompetitive with the nonspecific binding. These results suggest the possible involvement of a metal ion present in the receptor in the formation of the insulin-receptor complex.

Key words: Insulin-receptor, Zinc, Hormone-receptor interaction.

A possible functional relationship between zinc and insulin has been suggested after the observation made by QUATERMAN *et al.* (18) that rats subjected to a diet low in zinc exhibit a lower response to an intraperitoneal injection of insulin, and that the levels of plasma insulin are also lower than those of the control animals; ONER and BOR (17) have confirmed that such a diet leads to low levels of plasma insulin. KIM and LEE (12) showed that in healthy subjects taking zinc chloride, glucose injected intravenously tended to disappear from plasma more rapidly, while a delay in the disappearance of insulin was also observed. More recently COULSTON and DANDONA (2, 3) have described an activating effect of zinc on the lipogenesis in adipocytes, an effect which is parallel and synergistic with that of insulin. REUSS *et al.* (19) showed also

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that an increase in the velocity of binding of insulin to hepatocytes takes place in rats previously treated with zinc sulfate. ARQUILLA *et al.* (1) also observed that pretreatment of mice with Zn resulted not only in an accelerated binding of insulin but also in increased binding to liver plasma membranes compared to mice pretreated with Na; they also observed this latter effect *in vitro* when liver plasma membranes were incubated with iodoinsulin in the presence of added Zn. These data could suggest a possible implication of a metal in the binding of insulin to its receptor in the membrane. With the purpose of exploring this possibility, the metal content in purified receptors, as well as the effect of chelating agents, dialysis, and addition of metals to the medium on the binding of insulin to its specific receptor has been studied in rat liver plasma membranes, solubilized receptors, and purified receptors.

Materials and Methods

For the preparation of rat liver plasma membranes and the solubilization of receptors the method of CUATRECASAS (4) has been followed. Insulin receptors have been purified following the method of JACOBS *et al* (10). The purity of the receptor preparations was checked by SDS polyacrylamide gel electrophoresis (8).

Protein determination has been carried out with the technique of LOWRY *et al.* (14).

Metals have been determined by atomic absorption with a Perkin Elmer spectrometer, model 305-B. Hollow cathode lamps specific for each metal were used; metal concentrations were determined at the following wavelengths: 213.9 for Zn, 324.4 for Cu, 248.3 for Fe and 422.7 for Ca. Under these conditions the sensitivity of the method was between 0,10 μg/ml in the case of Zn for values of 1% of absorbancy, and with a within-day coefficient variation between 3.7% for Cu and 5.1% for Fe and a day-to-day coefficient of variation between 4.8% for Ca and 5.8% for Fe. Samples suspended in double distilled water were digested with nitric and sufuric acid (Suprapur, Merck) (1: 0.1: 0.1) for 12 h at 110°C, in sealed ampoules previously washed with Extran MA 02 (Merck).

When indicated, dialysis of the receptor preparations was carried out for 12 hours against 50 mM Tris-HCl buffer, pH 7.4, with or without either 5 mM, 1,10-phenantroline chloride (Merck) or 5 mM Zincon (2-carboxy-2' -hydroxy- 5' -sulfoformazylbenzene) (Sigma) for different periods, as mentioned in the description of the experiments. In order to remove the chelating agents, once the dialysis against them had been completed, a new dialysis against 50 mM Tris-HCl buffer was carried out for 24 hours changing the buffer 5 times.

The determination of total binding and specific Insulin-Receptor binding was carried out by the method of CUATRECASAS (4). [^{125}I] Iodo-Insulin (New England Nuclear) of 84-96 μCi/μg was used in all experiments. For the determination of total binding 50 μl of the suspension of plasma membranes or receptors containing approximately 1 mg protein per ml were incubated for 45 min in a medium containing 175 μl of labelled hormone solution. The separation of free hormone from that bound to protein was effected by adding polyethylene glycol and centrifuging at 6,000 × g for 30 min following the technique of KRUPP and LIVINGSTON (13). Free insulin remained in the supernatant. The radioactivity was measured in supernatants and precipitates with a 4,000 Beckman gamma counter. To calculate the nonspecific binding 10 μl of a non-labelled insulin solution were added to the medium prior to the addition of the labelled hormone to bring the ratio of non-labelled to labelled hormone to 100, and the samples were processed as

those used for total binding determination. Specific binding was calculated from the difference between total and non-specific binding.

Results

Kinetics of insulin-receptor binding. — Preparations of solubilized receptors were incubated in the presence of [^{125}I] Iodo-insulin in concentrations ranging from 1.3×10^{-10} M to 2.03×10^{-8} M. Saturation was reached at a hormone concentration of approximately 1.5×10^{-8} M. Double reciprocal plots of specific binding at different insulin concentrations gave a biphasic pattern (fig. 1). High affinity receptors were saturated at concentrations of the order of 10^{-9} M within the range of physiological insulin concentrations. The values of the dissociation constants calculated from the graph were 2×10^{-8} M and 6.4×10^{-10} M for the low and high affinity receptors respectively. The Hill coefficient calculated from these data has a value of 0.85.

Metal content of solubilized receptor, and purified receptor preparations. — Zn,

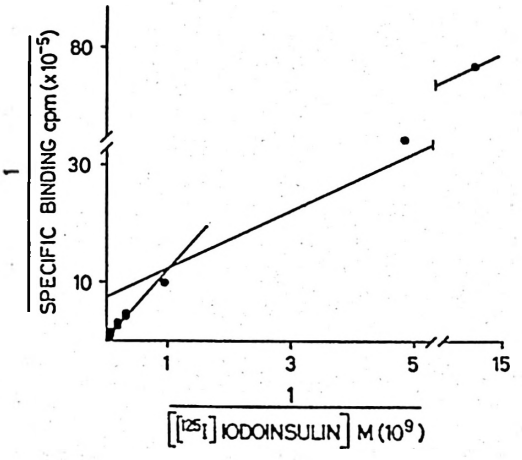

Fig. 1. *Double reciprocal plot of the specific binding of insulin to its receptor as a function of insulin concentration (n = 4).*

Table I. *Metal content (mμmoles/mg protein) in preparations of solubilized receptors.* Number of experiments has been indicated in each case in parentheses. Dialysis was carried out for 12 h against 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM 1,10-phenantroline.

	Zn	Cu	Fe	Ca
Before	2.3-6.0 (7)	0.35-1.0 (6)	3.4-10 (4)	2.7-61 (4)
After	0.9-1.8 (7)	0.14-0.7 (6)	0.4- 6.8 (4)	2.3-6.0 (4)

Cu, Fe and Ca were determined in different preparations of solubilized receptors. The metal content varied widely within the ranges indicated in table I. It may be observed that Cu was the most abundant metal. Some preparations were subjected to dialysis for 12 h against buffer containing 1,10-phenantroline. With the exception of Ca a substantial decrease in metal content was observed.

The Zn and Cu content was enriched in the process of purification of the receptor; at the end of the purification procedure the receptor had a calculated content of 3.3 atoms of Zn and 1.3 of Cu, assuming a M.W. of 300,000 for the receptor (5). However, Fe and Ca were not detected. The electrophoretic pattern of the purified receptor preparations was identical to that shown by JACOBS *et al.* (10). After dialysis for 12 h against 1,10-phenantroline the decrease in Zn and Cu was approximately 50%.

Effect of dialysis on insulin-receptor binding. — The effect of dialysis on the specific binding has been studied on different preparations of solubilized receptors. Dialysis of receptor preparations against Tris-HCl buffer for 12 h did not affect the specific binding and the metal content remained unchanged. However, when 1,10-phenantroline was present in the buffer a loss in metal content

accompanied by a decrease in specific binding was observed. A correlation exists between insulin binding and Zn or Cu content of solubilized receptor preparations after dialysis (fig. 2), either against buffer alone or against buffer plus the chelating agent. A 50% decrease in activity was observed in purified receptors after dialysis against 1,10-phenantroline.

When liver plasma membrane preparations were subjected to dialysis against buffer plus the chelating agent the specific binding was also affected; after 12 h the specific binding decreased in about 20% as compared to those dialyzed against buffer alone.

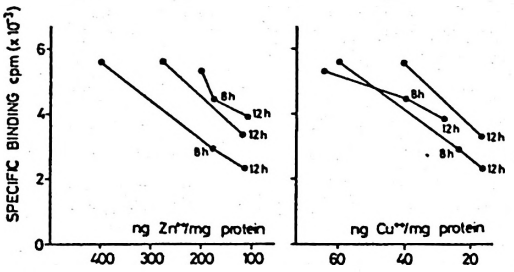

Fig. 2. *Relationship between hormone specific binding to its receptor and zinc or copper containing dialyzed receptor preparations.* [^{125}I]-Iodoinsulin concentration 2 × 10^{9} M. The number of hours of dialysis of each sample is indicated on each point.

Effect of the addition of metal ions to dialyzed and non-dialyzed preparations. The effect of the addition of a variety of metal ions to non-dialyzed preparations of receptors, and of plasma membranes, on the specific binding has been studied at different concentrations of insulin. Figure 3 shows how in preparations of solubilized receptors the addition of either Zn^{2+} or Cu^{2+} enhanced the specific bind-

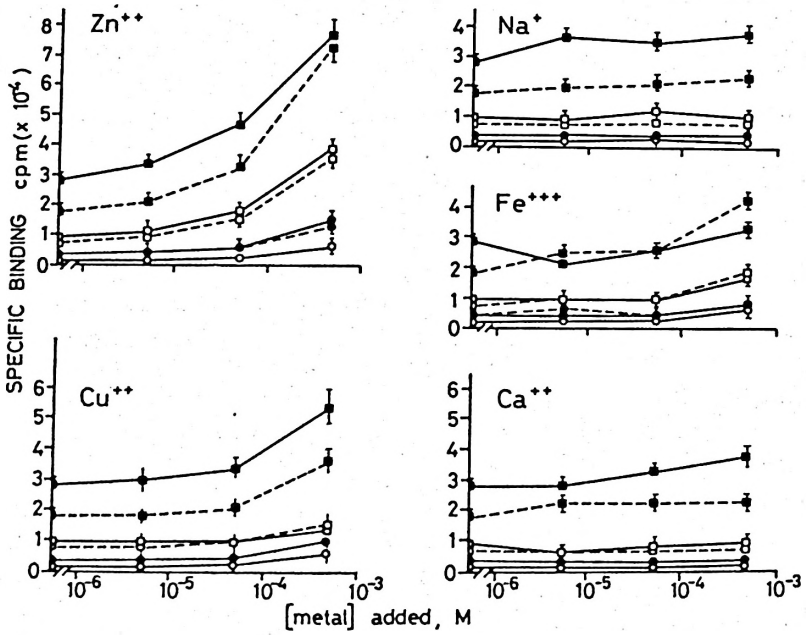

Fig. 3. *Effect of the addition of metal ions on the specific binding of insulin* [^{125}I]-*Iodoinsulin concentration:* 5 × 10^{-10} (○); 1 × 10^{-9} (●); 2.5 × 10^{-9} (□); 6 × 10^{-9} (■). Membrane suspension, ———; solubilized receptors, — — —. Cations were added as their chloride salts.

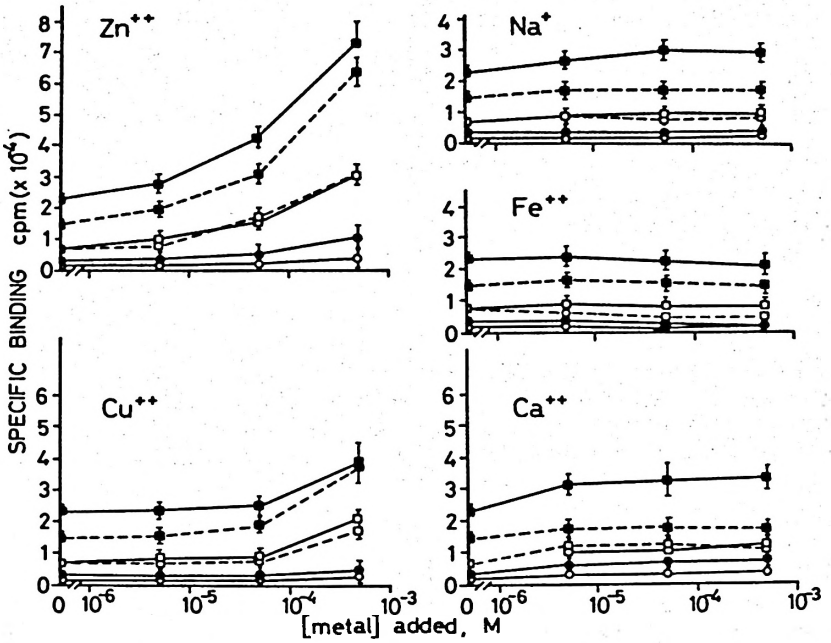

Fig. 4. *Effect of the addition of metal ions on the specific binding of insulin by dialyzed preparations.* Preparations were dialyzed against 5 mM 1,10-phenantroline, 50 mM Tris-Cl buffer for 12 h [^{125}I] -Iodoinsulin concentration: 5 × 10^{-10} (O); 1 × 10^{-9} (●); 2.5 × 10^{-9} (□); 6 × 10^{-9} (■). Membrane suspension, ———; solubilized receptors, — — —.

ing at any of the insulin concentrations used; however, no effect was observed when either Na^+ or Ca^{++} were the ions added; Fe^{3+}, on the other hand, only at concentrations higher than 5 × 10^{-5} M caused a slight increase in the specific binding.

The addition of either Zn^{2+} or Cu^{2+} to membrane preparations or solubilized receptors subjected to previous dialysis for 12 h resulted in a marked increase in the specific insulin binding (fig. 4); this increase was observed not only in those preparations dialyzed against 1,10-phenantroline, but also on those dialyzed against buffer alone. With 5 × 10^{-4} M concentrations of either Zn^{2+} or Cu^{2+} increases of up to fourfold the original value were observed. However, the addition of Ca^{2+}, Fe^{3+} or Na^+ to dialyzed preparatons did not affect the specific binding of insulin.

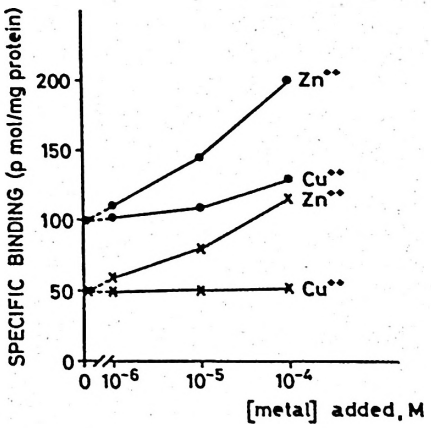

Fig. 5. *Effect of the addition of metal ions on the specific binding of insulin by purified receptors.* (●) Non-dialyzed receptors. (×) Receptors dialyzed against 5 mM 1,10-phenantroline, 50 mM Tris-Cl buffer for 12 h. [^{125}I]-Iodoinsulin concentration, 5.2 × 10^{-9} M.

With non preparations of purified receptor Zn^{2+} was the only ion leading to a clear enhancement of the specific binding; the effect of Cu^{2+} was very slight, and no effect was observed with either Ca^{2+} or Fe^{3+} (Fig. 5).

With preparations of purified receptors, dialyzed against chelating agents, which lost up to a 50% of specific binding, Zn^{2+} was the only metal capable of restoring the lost binding (fig. 5).

The effect of the addition of different metal ions on non-specific binding to dialyzed and non-dialyzed preparations of liver plasma membranes, and of solubilized or of purified receptors, was also studied. It was observed that whereas Na^{+} and Ca^{2+} had no effect, Fe^{3+}, Cu^{2+} and Zn^{2+} increased the non-specific binding in all preparations used, whether dialyzed or non-dialyzed.

Effect of preincubation of the receptor preparations in the presence of chelating agents. — Solubilized receptors were preincubated with 2.5 mM Zincon for 15 min prior to the addition of insulin at different concentrations. In the presence of Zincon the specific binding of the hormone exhibited a marked decrease at any of the insulin concentrations used (fig.

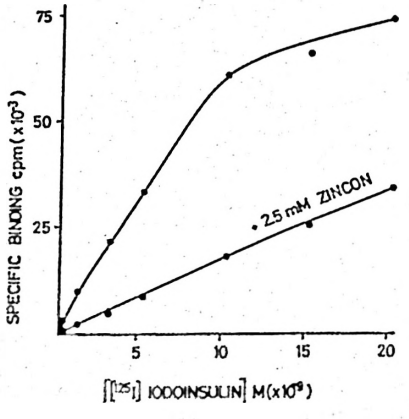

Fig. 6. *Effect of Zincon on the specific binding of insulin to its receptor. (n = 5).*

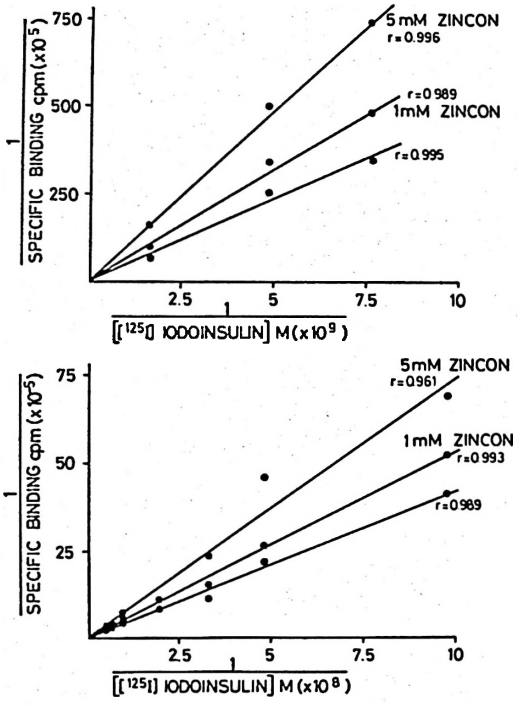

Fig. 7. *Double reciprocal plots of specific binding of insulin to its receptors versus insulin concentration in the presence of Zincon (n = 5).*

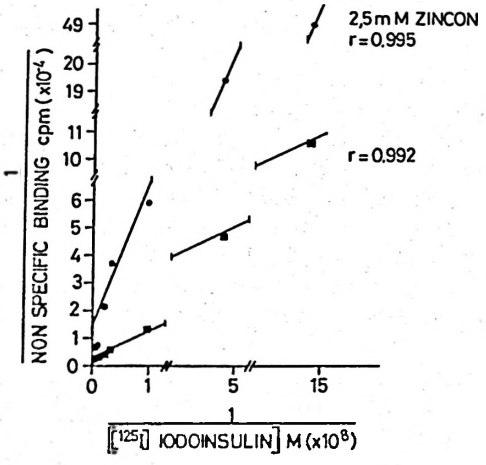

Fig. 8. *Double reciprocal plots of non-specific binding of insulin to its receptors versus insulin concentration in the presence of Zincon (n = 5).*

6). With the purpose of studying the nature of the effect of Zincon on the hormone-receptor interaction different concentrations of the chelating agent were added to preparations of the receptor in the presence of insulin at different concentrations. Figure 7 shows the double reciprocal plots of the specific binding versus insulin concentration ranging from 10^{-9} to 10^{-10} M, and from 3×10^{-8} to 10^{-9} M in the absence or in the presence of 1 or 5 mM Zincon. The intersection of the lines at the same point of the ordinate axis indicates that Zincon behaved as a competitive inhibitor of the hormone in its specific binding both to high and low affinity receptors. On the other hand non-specific binding was affected by Zincon in a non-competitive fashion (figure 8).

Discussion

The biphasic pattern of the double reciprocal plots of specific binding at different insulin concentrations could mean, as suggested by some authors (9, 11, 15), that two types of insulin receptors with different affinity are present, or as indicated by others (6, 7), that only one type of receptors exhibiting negative cooperativity exists. However, the value of 0.85 for the Hill coefficient found by us in dissociation studies of the insulin-receptor complex favors the second interpretation.

Metals Zn, Cu, Ca and Fe were present in variable amounts in solubilized receptors; in purified receptors only Zn and Cu were detected. Dialysis against buffer containing 1,10-phenantroline resulting in a loss of Zn, Cu and Fe (table I) led to a decrease in the specific binding of insulin. Different preparations of solubilized receptors (fig. 2) or of purified receptors subjected to dialysis for different periods showed a correlation between specific binding and their content in Zn and Cu. These results suggest the participation of a metal in the formation of the insulin-receptor complex, a view that received further support from the fact that chelating agent Zincon behaved as a competitive inhibitor of the specific binding of the hormone to its receptor (figs. 6 and 7), whereas it was noncompetitive of the non-specific binding (fig. 8).

It might be thought that the ionic environment of the hormone receptor could promote the binding of insulin to membrane proteins (5). However, the addition of Na^+ or Ca^{2+} to dialyzed and nondialyzed preparations, both of liver plasma membranes and of receptors, had no effect on specific binding (figs. 3 and 4), and the non-specific binding remained unaffected as well. The addition of Fe^{3+} resulted in an increase of the non-specific binding; however, the specific binding remained unaffected. At any of the concentrations of added Zn^{2+} or Cu^{2+} both the specific and non-specific binding increased. In preparations of purified receptors the specific binding was considerably enhanced by Zn^{2+}; the effect caused by Cu^{2+} was only very limited. These results agree with those previously reported by ARQUILLA *et al.* (1) on the effect of Zn^{2+} on non-specific binding; however, the elevation of the specific binding presented here took place at any of the concentrations tested, and not only at low Zn concentrations, as reported by ARQUILLA *et al.* The possibility of coprecipitation of insulin polymers with the insulin receptor complex is highly improbable, since at the assayed concentrations, insulin is present as monomer (19). The fact that the binding capacity lost by dialyzed preparations of either liver plasma membranes or receptors could be recovered by the readdition of Zn^{2+} suggests the possible involvement of this metal, present in the receptor, in the binding of the hormone. This could explain the activating effect of Zn, observed by COULSTON and DANDONA (2), on lipogenesis of adipocytes, or on that caused by insulin.

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Resumen

La adición de iones Zn^{2+} o Cu^{2+} a preparaciones de membrana plasmática o de receptor de insulina purificado de hígado de rata, origina un aumento del enlace específico de la hormona a su receptor; no se observa ningún efecto con la adición de Fe^{3+}, Ca^{2+} o Na^{+}. La diálisis, tanto de las preparaciones de membranas como de receptores purificados frente a agentes quelantes, como el Zincón (2-carboxy-2'-hydroxy-5'-sulfoformacilbenceno) o la 1,10 -fenantrolina, hace descender el enlace específico de la insulina, el cual aumenta tras la readición de Zn^{2+} o Cu^{2+} al medio. La adición de Ca^{2+}, Fe^{3+} o Na^{+} a preparaciones sometidas previamente a diálisis no afecta al enlace específico. La diálisis de la preparación del receptor purificado frente a agentes quelantes origina un descenso en el contenido de Zn^{2+} del mismo. El Zincón actúa como inhibidor competitivo de la insulina, interfiriendo con el sitio de enlace específico del receptor, y no competitivo en el enlace no específico. Estos resultados sugieren la posible implicación del ion metálico presente en el receptor en la formación del complejo insulina-receptor.

Palabras clave: Insulina, Receptores hormonales, Zinc.

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