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ORIGINALS

Receptor-Binding Assay of Chemically Modified Insulins

Comparison with in vitro and in vivo Bioassays

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Summary. The binding affinity for the insulin receptor was determined with a variety of insulin derivatives and compared to the biological activity *in vitro* and *in vivo* and to the physical properties of the derivatives. The relative binding affinity of each derivative was measured in a specific insulin-receptor binding system using mono ^{125}I-insulin and purified plasma membranes of rat liver. Twenty one chemically modified insulins were investigated, including acetylinsulins, crosslinked insulin dimer and insulin trimer, and insulins with an A_1—B_1 or A_1—B_{29} intra-molecular crosslink. The relative binding affinity corresponded to the relative biological potency *in vitro* for all of the derivatives studied. There was a good agreement between the activity *in vitro* and the physical properties as measured by circular dichroism spectroscopy with the acetylinsulins and with the crosslinked insulin monomer, dimer and trimer. In contrast, with most of the derivatives possessing an intra-molecular crosslink, the very reduced binding affinity (0.2—5.9%) and the comparably reduced biological potency *in vitro* opposed the moderate changes in physical properties. Biological activity was consistently higher *in vivo* than *in vitro*.

Key words: Receptor-binding assay of insulin, insulin receptor, plasma membrane, insulin derivatives, chemically modified insulins, biological activity *in vitro* and *in vivo*, insulin structure-function relationships.

The insulin specific binding system developed with mono ^{125}I-insulin [12] and purified liver plasma membranes [9—13] permits direct measurements of the affinity of an insulin analogue or of a chemically modified insulin for the insulin specific receptors. In earlier studies with insulins and insulin analogues that included proinsulin, A and B chains of insulin and insulins from various animal species, we observed that the ability to inhibit the ^{125}I-insulin binding to receptor was, in all cases, directly proportional to the biological activity *in vitro* [13].

In the present study, we have measured the affinity of chemically modified insulins for the insulin receptor in the liver plasma membrane. The derivatives investigated offer a wide range of chemical modification. Our purpose was to compare their binding affinity to their biological activity *in vitro* and *in vivo* and to their physical properties, as measured by circular dichroism (CD) spectroscopy. Some derivatives feature an intramolecular crosslink between the Gly A_1 α amino and the Lys B_{29} ε amino groups. Because these two amino groups are close to each other in the crystal structure [1, 2] and because the A_1—B_{29} crosslinked derivatives resemble proinsulin in several respects, it was of particular interest to see how such intramolecular crosslinks would affect the binding affinity for the insulin receptor.

Materials and Methods

Chemically Modified Insulin (Fig. 1)

Insulin derivatives were prepared by reacting beef insulin (obtained from Brunnengräber, Lübeck, 25 IU/mg) with mono- and bifunctional reagents and purified to give homogenous preparations. All substitutions were on the amino groups only. Amorphous standard beef insulin was obtained by gel filtration of the crystalline commercial product on Sephadex G-50 fine in 10% acetic acid, dialysis of the main fraction against water and reprecipitation of the lyophilized product from 0.005—0.01 N ammonia/HCl at pH 5.4.

The preparation of A_1, B_{29}-diacetylinsulin and A_1, B_1, B_{29}-triacetylinsulin has been described elsewhere [6]. Insulin monomer (A_1—B_1), dimer (B_1—B_1') and trimer (B_1—B_1', A_1'—B_1'') were prepared [5] with the bifunctional Edman reagent m-phenylene-diisothiocyanate (PBC).

Twelve derivatives intramolecularly crosslinked with aliphatic dicarboxylic acid residues—CO-(CH_2)m-CO— between the Gly A_1 α amino and the Lys B_{29} ε amino groups [3, 4, 7] were investigated. Depending on the total number of C-atoms ($n = m + 2$, ranging from 2 to 13) present in the crosslink, they realize various chain lengths (about 2—16 Å if fully extended) between the amino groups of glycine A_1 and lysine B_{29}.

Two derivatives were crosslinked with undecane ($n = 11$) and dodecane ($n = 12$) diacid residues between the N-terminal amino groups of Gly A_1 and Phe B_1.

Physical properties studied by CD spectroscopy and biological activity, as measured *in vivo* by blood sugar depression in rats [18] and *in vitro* by glucose oxidation in isolated fat cells [14], were determined on these insulin derivatives [3—7, 15].

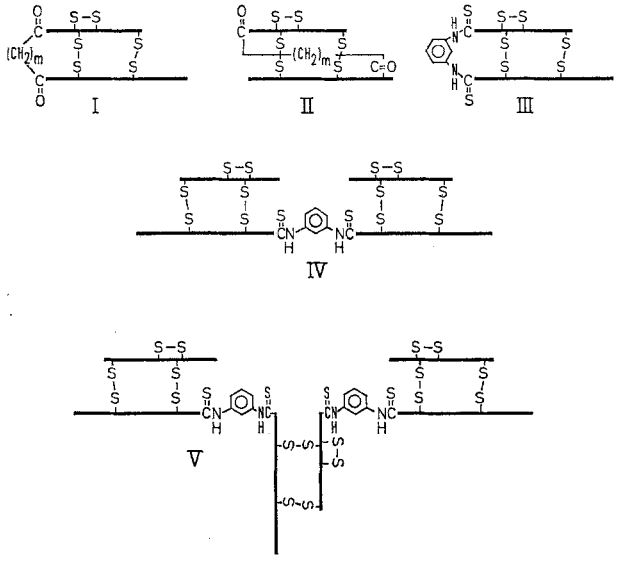

Fig. 1. Schematic representation of the structure of crosslinked insulin derivatives. I: N-terminal amino groups crosslinked with aliphatic dicarboxylic acid (m = 9, 10). II: amino groups of glycine-A_1 and lysine-B_{29} crosslinked with aliphatic dicarboxylic acid (m = 0, 2—11). III: A_1-B_1-PBC-insulin monomer. IV: B_1-B_1'-PBC-insulin dimer. V: B_1-B_1', A_1'-B_1''-PBC_2-insulin trimer

Receptor-Binding Assay of Chemically Modified Insulins

The insulin-receptor binding system consisted of mono ^{125}I-porcine insulin and purified plasma membranes from rat liver [9—13]. This *in vitro* system permits direct and quantitative measurements of the specific binding of insulin to its receptors with insulin concentrations as low as 0.05—1.0 nM. The inhibiting effects of insulin derivatives on the binding of ^{125}I-insulin to the plasma membrane were compared to effect of unlabelled native insulin.

Purified "mono component" porcine insulin (MCS 970, 27.2 IU/mg), kindly supplied by Dr J. Schlichtkrull (Novo Research Institute, Copenhagen, Denmark), was used for iodination and as the reference native hormone in the binding experiments. Mono ^{125}I-insulin was prepared and purified on DEAE cellulose as described previously [12], except that urea was omitted from the eluting buffer. Specific activities of approx. 350 μCi/μg ($\simeq$ 2000 Ci/mmole, or 0.9 I atom/mole of insulin) were achieved. Plasma membranes were prepared from rat livers according to Neville [16]; the fully purified plasma membrane fraction (step 15 in ref. 16) was used in all experiments.

Binding assays were performed at 30° C for 30 min[1] with membrane protein at 0.2—0.3 mg/ml incubation medium and 1.5% bovine serum albumin (Fraction V, Pentex), in Krebs Ringer phosphate buffer, pH 7.5; under these conditions, degradation of insulin [9] was less than 15%. ^{125}I-insulin at 0.1 nM was incubated with membranes in the absence and in the presence of various concentrations of unlabelled insulin or derivative (Fig. 2). The membrane-bound ^{125}I-insulin was isolated by rapid centrifugation at 4° C, as described previously [13].

Each derivative was tested at 3—6 concentrations within a single experiment and reassayed in 3—8 different experiments. Native insulin was tested (as the reference preparation) at least twice in any single experiment. The concentration of any given derivative that produced 50% inhibition of the ^{125}I-insulin binding was taken to express the binding affinity of the derivative relative to that of native insulin. Thus, the binding affinity of a derivative

$$= \frac{\text{molar concentration of porcine insulin}}{\text{molar concentration of derivative}} \text{ to achieve}$$

50% inhibiting effect $\times$ 100%.

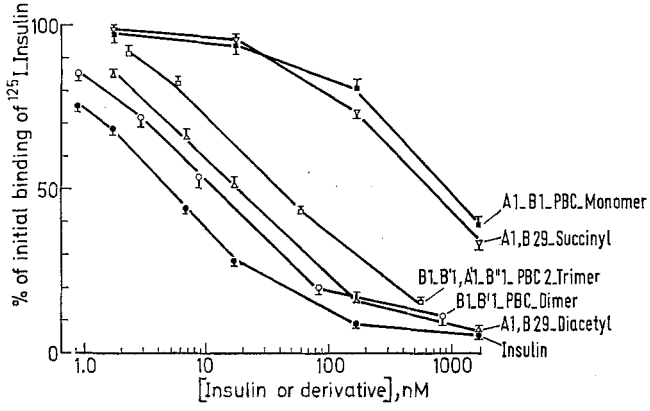

Fig. 2. Effect of native insulin and various insulin derivatives on binding of ^{125}I-insulin by liver plasma membranes. Binding is expressed as percent of initial (no unlabelled insulin or derivative added) binding of ^{125}I-insulin. This initial binding was approx. 20% of the total ^{125}I-insulin under the experimental conditions described in Methods. Each point is the mean ± SEM of 5—8 repeated experiments

Results

Data obtained in the receptor-binding assay are shown in Fig. 2. Comparison between the binding affinity and the biological activity is given in Tables 1 and 2.

1 Similar data were obtained when incubations were performed for 60 min

With the diacetyl and the triacetylinsulins, the reduction in binding affinity (25% and 8% that of native insulin, respectively) was similar to the reduction in biological potency *in vitro*, but contrasted with the apparent full retention of activity *in vivo*. It is of interest that the PBC-insulin dimer retained, on a monomer units in the trimer retains substantial activity.

Insulin derivatives that possess an intramolecular crosslink all exhibited a considerably reduced binding affinity. This was observed with the A_1—B_1 as well as with the A_1—B_{29} crosslinked derivatives. With the

Table 1. *Binding affinity and biological activity of insulin derivatives. All values are expressed as % of native insulin. Porcine insulin (27.2 IU/mg) and beef insulin (25 IU/mg) were the reference hormones in measurements of the binding affinity and of the biological activity, respectively*

Derivative		Binding affinity		Biological activity[a]	
		Mean	95% Conf. limits	In vitro[d] (Mean)	In vivo
A_1, B_{29}-Diacetyl		25	20 —31	28	100
A_1, B_1, B_{29}-Triacetyl		8	6 —10	24	100
B_1-B_1'-PBC-Dimer		46	34 —58	37	8
B_1-B_1', A_1'-B_1''-PBC_2-Trimer		13	8 —18	15	n.d.[c]
A_1-B_1 crosslinked	(n)[b]				
Undecane diacyl	(11)	0.2		0.9	5
Dodecane diacyl	(12)	0.2		0.6	4
A_1-B_1-PBC-Monomer		0.5	0.4— 0.6	1.2	< 1
A_1-B_{29} crosslinked	(n)[b]				
Oxalyl	(2)	1.1	0.8— 1.4	3.7	52
Succinyl	(4)	0.8	0.4— 1.2	2.7	30
Glutaryl	(5)	1.2	0.9— 1.5	5	32
Adipoyl	(6)	1.4	1.2— 1.6	5.2	42
Pimeloyl	(7)	1.5	0.9— 2.1	7.4	35
Suberoyl	(8)	1.5	1.2— 1.8	2.6	100
Azeloyl	(9)	1.8	1.4— 2.2	10.5	100
Sebacoyl	(10)	2.2	0.9— 3.5	10	61
Undecane diacyl	(11)	1.9	1.5— 2.3	12.5	69
Dodecane diacyl	(12)	2.3	1.9— 2.7	11.5	46
Tridecane diacyl	(13)	1.8	1.4— 2.2	8.5	40

[a] Data were obtained from references 4, 6 and 15
(n)[b] refers to the total number of C-atoms in the A_1-B_1 or A_1-B_{29} crosslink
n.d.[c] not determined
[d] Biological activity in vitro represents the relative potency of the derivatives; all of them showed the same maximal activity as insulin (see ref. 15).

Table 2. *Binding affinity and biological activity in vitro of suberoyl — and diaminosuberoyl — insulins. Data are expressed as indicated in Table* 1

	Binding affinity		Biological activity in vitro
	Mean	95% Conf. limits	(Mean)
Suberoyl	1.5	1.2—1.8	2.6
Diaminosuberoyl	5.9	4.7—7.1	4.4
$(Boc)_2$-diaminosuberoyl	1.0	0.6—1.4	n.d.
Diaminosuberoyl, reduced-reoxidized	5.2	3.1—7.3	n.d.

molar basis, almost 50% of the affinity and potency *in vitro* of the insulin monomer, suggesting that one of the two insulin molecules in this dimer is almost fully active. It is also remarkable that the PBC_2-insulin trimer, a rather large molecule, retained about 15% of the affinity and potency *in vitro* of the native insulin monomer, again indicating that at least one of the

latter, the relative binding affinity ranged from 0.8% with the succinyl to 5.9% with the diaminosuberoyl-derivative (Tables 1 and 2). The binding affinity increased slightly but significantly ($0.01 < p < 0.02$) with the length of the bridge from the succinyl to the sebacoyl derivative (Table 1). The increase in binding affinity was also significant ($0.01 < p < 0.02$) between the succinyl and the suberoyl derivative. In the latter, the A_1—B_{29} chain length corresponds, if fully extended, to the crystallographic distance (8—10 Å) between the connected sites [1]; however, the suberoyl-derivative had only 1.5% of the binding affinity of native insulin (Table 1).

It is of interest that the diaminosuberoyl-derivative exhibited a higher binding affinity than that of the suberoyl-derivative (Table 2). The diaminosuberoyl-insulin had the highest relative binding affinity (5.9%) amongst all of the A_1—B_{29} crosslinked derivatives studied. Blocking the free amino groups of the diaminosuberoyl-derivative with butyl-oxy-carbonyl (Boc)

decreased the binding affinity down to that observed with the suberoyl-derivative (Table 2), indicating that free amino groups may be important, either directly or indirectly, in the receptor-binding process. Reduced-reoxidized diaminosuberoyl-insulin was almost as active as diaminosuberoyl-insulin (Table 2). This finding complements, at the membrane receptor level, recent observations with adipoyl-insulin which suggest that A_1—B_{29} crosslinked derivatives behave similarly to proinsulin with respect to the ability to readopt the original conformation after reduction and reoxidation [7].

With all of the intramolecularly crosslinked derivatives, the relative binding affinity was either in close correspondence or, in most instances, lower than the relative biological potency *in vitro* (Tables 1 and 2). In contrast to *in vitro* data, the biological activity of the A_1—B_{29} crosslinked insulins appeared to be largely retained *in vivo* (Table 1).

Discussion

Since most of the chemically modified insulins used in this study presented a very reduced biological potency *in vitro*, it was of interest to examine directly, in the insulin receptor-binding system, the possibility that some of them might have retained a binding affinity "in excess" of their reduced biological potency and could thus behave as antagonist(s) of insulin. This was not found to be the case, and all of them appeared to behave as insulin agonists of lower potency. These data thus extend to the various derivatives studied here our previous findings with other insulins and insulin analogues [10, 13]. They also indicate that the changes induced by the present chemical modifications do not disclose discrete regions of the structure that would be separately responsible for binding to receptor and for activating cellular processes once the hormone-receptor complex has been formed.

There was a good agreement between the data obtained *in vitro* and the physical properties as studied by CD spectroscopy [4, 5] with the acetylinsulins and with the insulin monomer, dimer and trimer. In contrast, the A_1—B_1, and even more strikingly the A_1—B_{29} crosslinked derivatives all exhibited a very reduced binding affinity and biological potency *in vitro* that opposed the moderate changes in their CD spectra [4]. A_1—B_{29} crosslinked insulins resemble proinsulin in several respects: they behave as insulin agonists of very low potency *in vitro* and are capable of readopting their original properties after reduction and reoxidation (Table 2 and ref. 7). It is possible that, somewhat similarly to the connecting peptide in the proinsulin molecule [11], the aliphatic crosslink between Gly A_1 and Lys B_{29} may alter or hinder, in the vicinity of Gly A_1, a region of the insulin molecule [1, 2] which is likely to be involved in binding to the receptor. In addition, or alternatively, such a link could impede a conformational change possibly required for binding to the receptor.

The discrepancy between the loss of activity *in vitro* and the apparent retention of activity *in vivo* is another striking feature of the A_1—B_{29} crosslinked insulins. *In vitro* studies allow one to measure the affinity for the receptor, and the potency and the efficacy throughout a wide range of concentration of the hormone or derivative; degradation of the hormone or derivative can be minimized or controlled. *In vivo* data reflect an overall effect which results from several processes: in addition to the potency and the efficacy, distribution and degradation may differ amongst the various derivatives when compared to insulin. For example, the slower degradation rate of proinsulin appears to be responsible, at least in part, for its longer half-life [17] and prolonged activity *in vivo* [8], and may account for its higher relative activity *in vivo* than *in vitro* when compared to insulin. It is possible that similar differences can explain the higher activity *in vivo* than *in vitro* observed here with the A_1—B_{29} crosslinked derivatives.

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