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BIOLOGICAL POTENCY AND BINDING AFFINITY OF MONOIODOINSULIN WITH IODINE IN TYROSINE A14 OR TYROSINE A19

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SUMMARY: Monoiodoinsulin with 99% of the iodine in the A chain was separated in two bands by disc-electrophoresis using long polyacrylamide gel rods. The bands contained monoiodoinsulin with the iodine in tyrosine A14 and tyrosine A19, respectively. The biological potency and binding affinity of A14 [^{127}I]monoiodoinsulin on rat adipocytes was indistinguishable from that of insulin, whereas the A19 derivative was only half as potent. Similarly, the maximum binding of "tracer" A19 [^{125}I]monoiodoinsulin to adipocyte receptors was only half of that obtained with A14 [^{125}I]monoiodoinsulin.

INTRODUCTION:

Monoiodoinsulin is biologically active as first shown by Freychet et al. (1) and later confirmed by others (2-6). Some authors have found the biological potency of monoiodoinsulin indistinguishable from that of insulin (1,5,7) whereas others have found a slightly reduced potency (2,4,6). Previous studies from our laboratories showed a significantly reduced potency and binding affinity on isolated rat adipocytes of a monoiodoinsulin preparation with 99% of the iodine in the A chain and of this 30% in tyrosine A14 and 70% in tyrosine A19 (4,6). Studies of the structure-function relationship of various insulin analogues

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(8) have suggested that tyrosine A19 is near the putative receptor binding region and iodination of this residue might therefore reduce the binding affinity. The iodoinsulin preparations studied until now have been heterogeneous with respect to location of iodine in the A chain. Since [^{125}I]iodoinsulin preparations are widely used as tracers for insulin we decided to study the biological properties of A14 and A19 monoiodoinsulin.

METHODS:

Highly purified insulin was obtained from Nordisk Insulinlaboratorium. [^{127}I]iodoinsulin containing a trace amount of [^{125}I]iodoinsulin was prepared using the lactoperoxidase method (3) or the triiodine method (4). Monoiodoinsulin was separated from insulin and more highly iodinated molecules by ion exchange chromatography using AE cellulose as described previously (4). Monoiodoinsulin and control insulin were weighed out and dissolved in 1 mM HCl to a concentration of 150 μM. The correctness of the estimated concentrations was controlled by measuring optical density at the absorption maxima (5) and by determination of the protein concentrations (9). The specific activity of two preparations of monoiodoinsulin (lactoperoxidase method) was determined as 12.13 and 11.27 Ci/mol, i.e. about 0.06% of the molecules contained [^{125}I]. The iodine content in the B chain was determined as 1.5-4.2% after oxidative sulphitolysis (4,10). The iodine distribution in the A chain was determined as 15-30% in tyrosine A19 and 85-70% in tyrosine A14 after chymotryptic hydrolysis (4,11) as will be described in detail elsewhere (Linde and Hansen, manuscript in preparation). Monoiodoinsulin prepared using the triiodine method contained 99% of the iodine in the A chain and of this 70% in A19 (4). The biological potencies of the monoiodoinsulin preparations, determined by the method of Moody et al. (12) as modified for the use of ^{125}I-labelled analogues (13), were found to be slightly lower than that of insulin in agreement with previous results (4,6).

Samples of about 4 nmol monoiodoinsulin were separated in two bands by disc-electrophoresis in polyacrylamid gel using long gel rods as described previously (4). The position of the iodine in the two bands was analyzed and it was found that one of them (band III of ref. 4) contained essentially only A14 monoiodoinsulin, whereas the other one (band II of ref. 4) contained A19 monoiodoinsulin plus the small amount of monoiodoinsulin with the iodine in the B chain. Neither of the bands contained insulin or iodoinsulin with more than one iodine per molecule. Staining with Coomassie Brilliant Blue (14) showed that practically all protein was in bands II plus III (maximum contamination with insulin was 3%). Equivalent unstained gels were sliced and the gel pieces corresponding to bands II and III were eluted. The concentrations of monoiodoinsulin were calculated from the concentrations of radioactivity in the eluates and the precisely determined specific activity of the unfractionated monoiodoinsulin. This calculation is based on the assumption that

the specific activity of the A14 and A19 monoiodoinsulin derivatives is identical in a given preparation of monoiodoinsulin. The biological potencies and binding affinities of the eluted monoiodoinsulin preparations were determined according to previously published methods (12,13).

[^{125}I]monoiodoinsulin was prepared using lactoperoxidase essentially as described by Sodoyez et al. (5). 9-17 nmol insulin was iodinated with 5 mCi [^{125}I] with a specific activity of 2.1 mCi/natom as indicated by the manufacturer (New England Nuclear). The monoiododerivatives were separated from insulin by disc electrophoresis as described above and 70% was A14 monoiodoinsulin. Two other iodoinsulin preparations of high specific activity were subjected to gel electrophoresis. Iodoinsulin prepared by the iodate method (15) (commercially available from NOVO Research Institute) contained 6% of the iodine in the B chain, 94% in the A chain and of this 90% in A19 and 10% in A14. Iodoinsulin prepared by the chloramin-T method (16,17) contained 19% of the iodine in the B chain, 81% in the A chain and of this 15% in A19 and 85% in A14. The bands were eluted and the biological properties of the materials were studied.

RESULTS:

Figure 1 shows that the A14 [^{127}I]monoiodoinsulin prepared by the lactoperoxidase method (about 70% in A14 and 30% in A19) as well as the A14 [^{127}I]monoiodoinsulin prepared by the triiodine method (about 30% in A14 and 70% in A19) exhibited the same biological potency as insulin. On the other hand, the A19 derivative prepared by either methods was only half as potent.

Figure 2 shows that insulin and the two preparations of A14 monoiodoinsulin competed equally well with [^{125}I]monoiodoinsulin of high specific activity for binding to adipocyte receptors, whereas approximately twice as high concentrations of the A19 monoiodoinsulin preparations were needed to cause the same displacement.

The biological activity of the A14 [^{125}I]iodoinsulin of high specific activity was determined on isolated rat adipocytes and the concentrations were determined according to the assumption that this derivative has the same potency as insulin, cf. Figure 1. The specific activity was then calculated as about 1.3 mCi/nmol in 5 preparations. This is somewhat lower than the

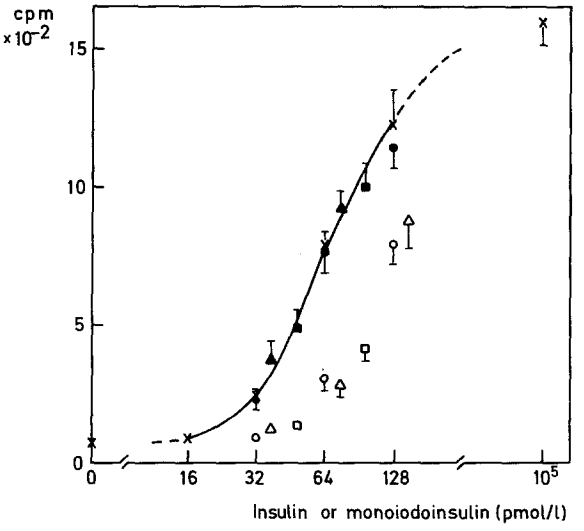

Figure 1. Biological potency of A14 and A19 monoiodoinsulin. Isolated rat adipocytes (4 µl packed cell volume) were incubated for 2 h in 1 ml Krebs-Ringer HEPES (10 mM) buffer (37 °C, pH 7.4) with albumin (10 mg/ml), [U-^{14}C]glucose (0.05 µCi/ml) and insulin or monoiodoinsulin as indicated. The incubation was terminated by the addition of 10 ml toluene-based scintillation fluid and the samples were allowed to stand at 4 °C over night to extract the ^{14}C-lipids. 5 ml of the organic phase was then transferred to other vials and counted. The effect of insulin on the rate of synthesis of ^{14}C-lipids is shown by the crosses. The filled symbols represent band III material (A14 monoiodoinsulin) either from monoiodoinsulin prepared by the lactoperoxidase method (●-● and ▲-▲ represent two different preparations) or by the triiodine method (■-■). The open symbols represent the corresponding band II material (A19 monoiodoinsulin contaminated with 4-12% B chain-substituted monoiodoinsulin). The bars represent SD, n = 4.

specific activity of the iodine indicated by the manufacturer (2.1 mCi/nmol). However, re-electrophoresis did not increase the specific activity of the monoiodoinsulin and it seems likely that the manufacturer's estimate is too high. In some experiments the potency (i.e. U/Ci) was determined on A14 and A19 derivatives from the same iodination (iodate method) and the potency of the A19 derivative was consistently determined as about half of that of the A14 derivative (data not shown).

The binding affinities of the A14 and A19 [^{125}I]monoiodoinsulins were compared by determination of the maximal ratio:

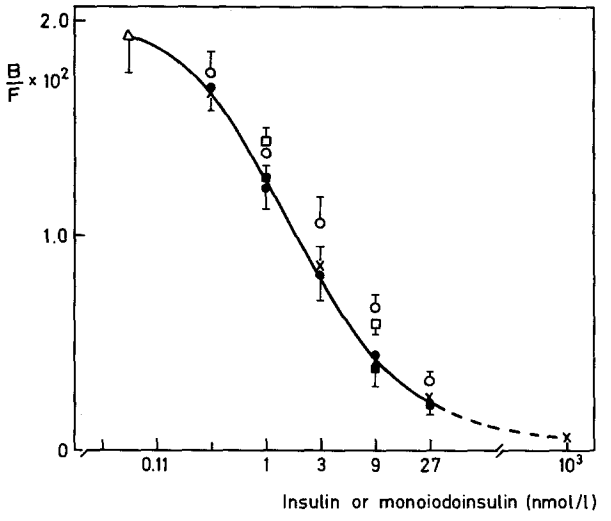

Figure 2. Binding affinity of A14 and A19 monoiodoinsulin. Adipocytes (24 µl packed cell volume) were incubated in 0.5 ml Krebs-Ringer HEPES buffer with albumin 50 mg/ml and 80 pM [^{125}I]monoiodoinsulin in 14 ml polypropylene tubes. pH was 7.4 and the incubation time 40 min at 37 ^{0}C, i.e. to steady state of binding. The incubations were terminated by the addition of 10 ml chilled (10 ^{0}C) 0.9% NaCl followed by 1.2 ml of silicone oil (density 0.99, viscosity 100 centistokes). The tubes were centrifuged for 40 s at about 1000 x g, and the cells were recovered on absorbent material from the top of the oil phase as described previously (13). Tracer alone (Δ). Tracer plus insulin (x-x). The meaning of open and filled circles or squares is the same as indicated in the text to Figure 1. Bars represent SD, n = 4.

bound hormone/free hormone. When the concentration of the "tracer" is very much lower than the dissociation constant for insulin binding on adipocytes, the ratio: bound/free becomes identical with the ratio: concentration of receptors/dissociation constant for the labelled ligand, i.e. the maximal ratio: bound/free (18). Therefore, when experiments are carried out on the same cell pool (same receptor concentration), variations in this ratio will reflect variations in the dissociation constant.

Figure 3 shows that about 2% of the A14 derivative was bound whether obtained from monoiodoinsulin prepared using lactoperoxidase, iodate or chloramin-T. On the other hand, only half as much was bound of the A19 derivative obtained from the mono-

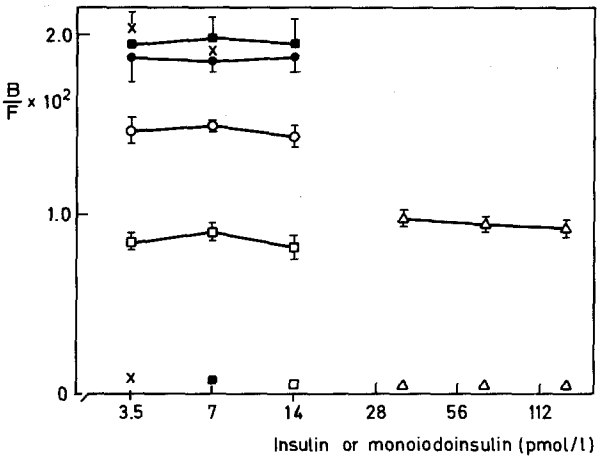

Figure 3. Binding of A14 and A19 [^{125}I]monoiodoinsulin. Adipocytes were incubated with [^{125}I]monoiodoinsulins as described in the text to Figure 2. The low concentrations were chosen to ensure that B/F was maximal. The open triangles show the binding of the mixed preparations (iodate method) obtained from NOVO. The specific activity was 0.15 mCi/nmol as indicated by the manufacturer (and controlled by bioassay), i.e. about 90% of the protein content was insulin. The other symbols refer either to the band III material (A14 monoiodoinsulin) from gel electrophoresis of the preparations obtained using iodate (■-■), lactoperoxidase (●-●) or chloramin-T (x-x), or to the equivalent band II material (iodate method (□-□), lactoperoxidase method (o-o)). For calculation of concentrations the specific activities were approximated as 1.3 mCi/nmol. The distribution of iodine in A19/B chain was 94/6 in band II monoiodoinsulin prepared by the iodate method and 69/31 in band II monoiodoinsulin prepared by the lactoperoxidase method. Binding of band II monoiodoinsulin prepared by the chloramin-T method is not shown since it contained 61% of the iodine in the B chain. The symbols with very low B/F values (<0.1 x 10^2) indicate binding of tracer in the presence of 10^{-6} M insulin. The bars represent SD, n = 4.

iodoinsulin prepared with iodate. The tracer of the mixed "iodate" preparation also exhibited low binding in accordance with the distribution of iodine in A14/A19. The band II material of the "lactoperoxidase" preparation of high specific activity showed an intermediate position. The reason is presumably that 31% of the iodine in that band was in the B chain (predominantly B16).

DISCUSSION:

We have shown that A14 monoiodoinsulin exhibits the same binding affinity and potency as insulin whereas A19 monoiodo-

insulin only has about half of the affinity and potency on adipocytes. A large number of insulin analogues have shown parallel changes in the relative affinity to receptors of adipocytes and receptors of other cell types, e.g. liver cells (19,20). We have also observed that the maximal binding of A14 [^{125}I]monoiodoinsulin in hepatocytes and cultured human lymphocytes of the IM-9 line is about twice as high as that of A19 [^{125}I]monoiodoinsulin (Sonne and Gliemann, unpublished observation). It is therefore likely that A19 monoiodoinsulin will exhibit decreased affinity to insulin receptors in general. Consequently, "tracer" monoiodoinsulin (iodate method (14), horseradish peroxidase method (21)) will bind less well than "tracer" prepared by methods yielding largely A14 monoiodoinsulin (lactoperoxidase method (3), chloramin-T method (16,17)).

The use of tracer with decreased affinity will bias not only *in vitro* receptor studies as discussed previously (6), but probably also studies concerned with the metabolism of insulin *in vivo* (22). The use of "tracers" containing both A14 and A19 monoiodoinsulin will add to the complexity of the experimental system and should be avoided when possible. Iodination as described in the present report followed by electrophoretic separation of A14 monoiodoinsulin from other iodinated derivatives and insulin is a convenient and effective method for preparation of a homogeneous "tracer" with high specific activity and with the same receptor binding affinity as that of insulin.

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