Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 541-549

THE PRESENCE OF LIPOPHILIC GLYCOPROTEIN INTERACTING WITH INSULIN

Takashi Momoi

Department of Biochemistry, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, (Japan)

Received February 15,1979

Summary

A lipophilic glycoprotein with insulin-binding activity could be extracted with organic solvent from rat liver. After removal of lipid, the addition of Concanavalin A, or treatment with trypsin or neuraminidase caused a decrease in the insulin -binding, which showed a complex kinetics. After purification by Concanavalin A- and insulin-Sepharose affinity column chromatographies, the fraction having insulin-binding activity exhibited two protein bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Introduction

Integral membrane glycoproteins are strongly associated with lipids by their hydrophobic cores (1). The possible relation of membrane lipids to the organization and the enviroment of the insulin receptor has been studied (2-5). The

---

Abbreviations

PBS : phosphate balanced saline buffer.
Tris-HCl buffer : 50 mM Tris-(hydroxymethyl)-aminomethane hydrochloride buffer (pH 7.4).
THN buffer : 50 mM Tris-HCl buffer containing 0.1 % Nonidet P-40 (pH 7.4)
CMW : chloroform-methanol-water (30 : 60 : 8, v/v/v)
NP-40 : Nonidet P-40
SDS : sodium dodecyl sulfate
Con A : Concanavalin A
Egg PC : egg phosphatidyl choline
Chol : cholesterol
BSA : bovine serum albumin
WP : water phase
IP : interphase
OP : organic solvent phase

Present address
c/o Professor Dr. H.Wiegandt, Institut für Physiologisch-Chemie, Philipps Universität, D-355 Marburg, Lahnberge, FRG.

0006-291X/79/060541-09$01.00/0

insulin effects were mimicked by the treatment with phospholipase C or A, which altered the conformation of membrane lipoprotein (2-4). The presence of " occult receptor " which was masked by lipids, was suggested by the observation that insulin-binding activity was enhanced by the treatment of membranes with phospholipase C or ether-ethanol (3 : 1, v/v) extraction, but not with ether or chloroform alone (5). It was not clear why the enhancement by ether-ethanol was less than by enzyme digestion, despite the fact that the membranes after the treatment with ether-ethanol were not affected by the treatment with phospholipase C (5). One explanation is that some insulin receptors were extracted with the organic solvents together with lipids, although alternative possibilities, e.g. denaturation by organic solvent, still remain. If indeed such a lipophilic receptor could be shown, it may conceivably account for the process of the internalization of insulin into the cell.

Therefore, this study was initiated to detect an insulin-binding substance in the ether-methanol (1 : 1, v/v) extract of liver membranes. This paper describes the isolation and the characterization of such lipophilic insulin-binding substance.

Methods and materials

Isolation

Pooled male Wister-strain rat liver (wet weight, 350 g) was minced and washed five times with 10 vol. ice-cold PBS, and homogenized in a warling blender. After the extraction with a large amount of cold acetone (4°C), the residue was extracted with 10 vol. of ether-methanol (1 : 1, v/v) at room temperature overnight, and filtered. The filtrate was concentrated to about 50 ml of viscous lipidic solution by rotary evaporation at 37°C, avoiding complete dryness. The insulin-binding activity in the extract was eluted from DEAE-Sephadex A-25 (acetate form) column (2 X 30 cm) with ammonium acetate, 0.05 M and 0.1 M each in 100 ml of chloroform-methanol-water (30 : 60 : 8, v/v/v) (CMW) (6). After the removal of ammonium acetate by Sephadex LH-20 column (2 X 30 cm), the sample was diluted and subjected to rechromatography on DEAE-Sephadex A-25 column (acetate form) (2 X 30 cm). Elution was carried out with a linear gradient of ammonium acetate from 0 to 0.2 M in CMW. The

fractions with insulin-binding activity were combined and dialyzed against a large volume of Tris-HCl buffer at 4°C overnight. The sample was separated into three phases during dialysis, water phase (WP), interphase (IP), and organic solvent phase (OP). Insulin-binding activity was mainly detected in IP (about 20 %) and OP (about 80 %). OP was evaporated nearly to dryness under a nitrogen stream at 0°C, and ethanol was rapidly added to the wet residue (the final volume, 200 µl). The sample was passed through a Iatrobeads, porous silica gel, (Iatro Laboratories Co., Tokyo) column (0.5 X 3 cm) (6). Elution was carried out with ethanol at 4°C. The delipidated insulin-binding substance (fraction 18 to 20 in Fig. 2) was dialyzed against Tris-HCl buffer containing 0.1 % NP-40 (THN buffer) at 2°C after addition of 0.01 % (w/v) egg PC and 0.1 % (w/v) NP-40. The characterization of this binding substance was performed as described below.

Affinity column chromatography

Con A-Sepharose 4 B (Pharmacia Fine Chemicals) column (0.5 X 2 cm) was equilibrated with THN buffer. The sample was applied and washed with THN buffer at 4°C. Bound materials were then eluted with 1 M methyl-α-D-mannoside at room temperature. Insulin-Sepharose (6 µg/mg packed weight) was prepared by coupling amino group of lysin residue of insulin with activated CH-Sepharose (Pharmacia Fine Chemicals) in 0.1 M $NaHCO_3$ (pH 9.0) as described by Cuatrecasas (7). Bound materials eluted from Con A-Sepharose column were applied to the insulin-Sepharose column (0.5 X 2 cm) equilibrated with 50 mM phosphate buffer (pH 7.4) containing 0.1 % NP-40. Then the column was washed with the same buffer containing 0.5 % NaCl at 4°C, and insulin-binding substance was eluted with 4.5 M urea in 50 mM acetate buffer (pH 6.0) containing 0.1 % NP-40 at room temperature. Fractions were immediately diluted with an equal volume of 50 mM phosphate buffer (pH 7.4) to avoid the time dependent denaturation by urea.

Assay of insulin-binding activity

Insulin-binding activity in the presence of a large amount of lipids was assayed in the liposome suspension. Five or 10 µl of each fraction eluted from the DEAE-Sephadex A-25 and Sephadex LH-20 column was evaporated under nitrogen stream short of complete dryness and in the presence of 5 µl of ethanolic solution containing 0.1 % (w/v) egg PC added as an auxillary lipid. The amount of phospholipid in each aliquot was less than the auxillary egg PC added. The residue was dissolved in 5 µl of ethanol, and 1 µl of each was rapidly injected in 100 µl of Tris-HCl buffer through a thin needle microsyringe (Termo microsyringe) to prepare liposomes (8). The assay mixture (100 µl) was ; 50 µl of liposome suspension, 0.1 % (w/v) BSA, and 0.7 nM [^{125}I]insulin (41 mCi/mmol) (Dinabot Chemicals). The mixture was incubated in the presence or absence of native insulin (27 µM) at 37°C for 1 h. The binding was stopped by addition of 1 ml ice-cold Tris-HCl buffer, then mixture was rapidly filtered through a Millipore filter (EGWP, pore size, 0.2 µm). The filter was then washed with an additional 10 ml of the same chilled buffer, and submitted to counting of its radioactivity. Insulin-binding activity was obtained by subtracting the radioactivity that was not displaced by native insulin from the total radioactivity. The presence of auxillary lipids, egg PC (1 µg/100 µl) in the assay mixture, is indispensable not only stabilizing the insulin-binding

activity, but also for keeping the filtration rate through the Millipore filter constant, and for eliminating any variables that might be accompanied with preexisting lipids in samples. Egg PC alone (1-10 μg/100 μl) did not show any significant insulin-binding activity. Because these water soluble insulin-binding substances could not be trapped on Millipore filter, all the eluates from the Iatrobeads column, Con A- and insulin-Sepharose column were assayed according to the polyethylene glycol method developed by Cuatrecasas (9). This method was slightly modified as described below to obtain constant and a high filtration rate (15-30 sec). Binding was stopped by the addition of 1 ml of ice-cold Tris-HCl buffer containing 8 % (w/v) polyethylene glycol 6000. After the rapid filtration through the Millipore filter (EAWP, pore size, 1.0 μm), the filter was washed with an additional 10 ml of the same buffer.

Specificity of insulin-binding substance

Purified sample was incubated with trypsin (EC 3.4.4.4) or neuraminidase (EC 3.2.1.18) (protease free, from Arthrobactor, Nakarai, Tokyo) (10), at various concentrations at 37°C for 30 min. Trypsin treatment was terminated by adding trypsin inhibitor four fold the amount of trypsin. These enzyme-treated preparations were incubated with 0.7 nM[^{125}I]insulin without removal of enzymes and inhibitor at 37°C for 1 h. Insulin-binding activity was assayed by the polyethylene glycol method as described above.

Con A agglutination activity

Liposomes were prepared from each fraction and egg PC-Chol (2 : 1, w/w) by the method as described above (8,11). Liposomes were incubated with Con A (1 mg/ml) at 37°C for 1 h with or without 1 M methyl-α-D-mannoside.

SDS polyacrylamide gel electrophoresis in semimicroscale

After removal of NP-40 from the sample by Bio Beads SM-2 (Bio Rad Lab.) (12), the sample was boiled in the presence of 1 % SDS and 1 % 2-mercaptoethanol for 1 min, and applied to a cylindrical polyacrylamide gel (T=4 % to 40 % gradient gel, C=2 %) (0.8 mm X 3 cm). Electrophoresis was carried out at a constant voltage of 30 V. Proteins were stained with Coomasie Blue R-25. This system could detect 10 ng of protein (13)

Determination of protein and inorganic phosphate

Protein was determined by a modification of Lowry's method (14,15) and fluorescamine method (16) using a crystalline BSA as a standard. Inorganic phosphate was determined by the method of Bartlett (17).

Results and discussions

The insulin-binding activity which was detected in ether-methanol (1 : 1, v/v) extracts from rat liver could be eluted from DEAE-Sephadex A-25 (acetate form) column with 0.05 to 0.1 M ammonium acetate after the removal of most of the non-anionic lipids and nonspecific binding substances interacting with insulin by ionic strength. Each fraction was tested for

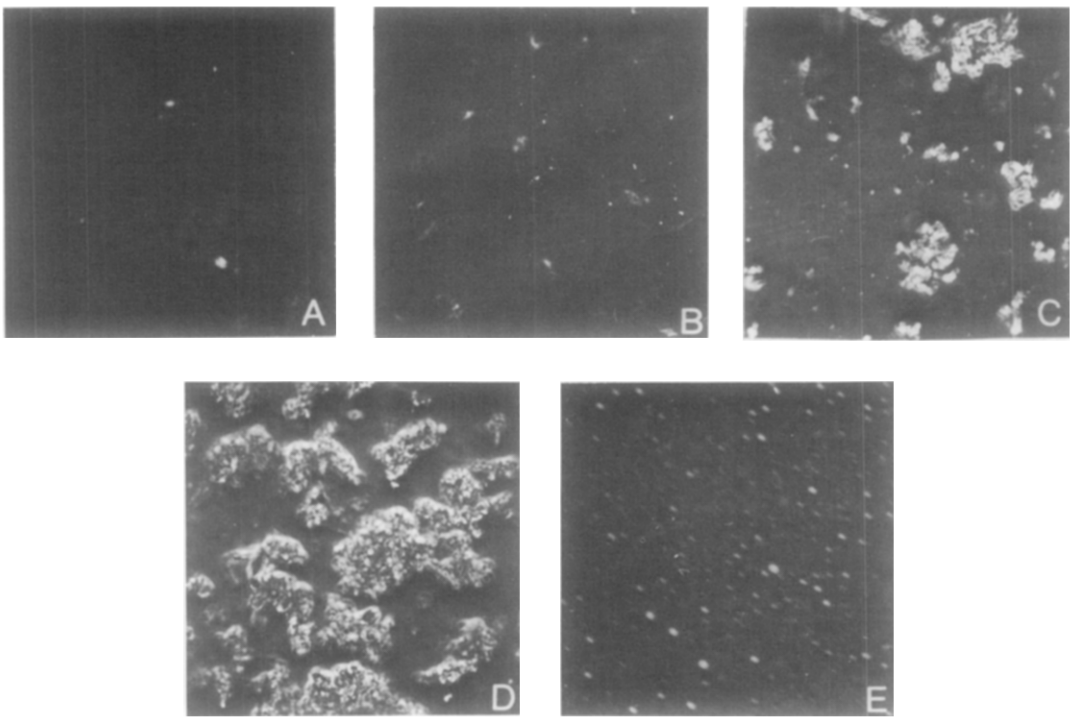

Fig. 1. The liposomes were prepared from WP, IP and OP as described in Methods. Liposomes from WP were prepared after lyophilization of aliquot of WP. Each liposome suspension (450 µl) was incubated with Con A at a concentration of 1 mg per 1 ml at 37°C for 1 h. The agglutination was examined under a phase contrast microscope (X 400). Numbers in parenthesis indicate the insulin-binding activity of 1 ul aliquot of each fraction (final volume was adjusted to 200 ul with ethanol).
A : egg PC and Chol liposome, B; WP, egg PC and Chol liposome, C; IP, egg PC and Chol liposome, D; OP, egg PC and Chol liposome, E; inhibition by 1 M methyl-α-D-mannoside of agglutination of WP with Con A.

Con A agglutination as well as insulin-binding activity, because Con A is known to bind specifically with the insulin receptor of biomembranes (18-20). A specific insulin-binding activity, together with Con A agglutination activity was detected in the organic solvent phase (IP and OP) rather than in the water phase (WP) (Fig. 1). Most of the lipids in the OP were removed by the Iatrobeads column chromatography. A part of the

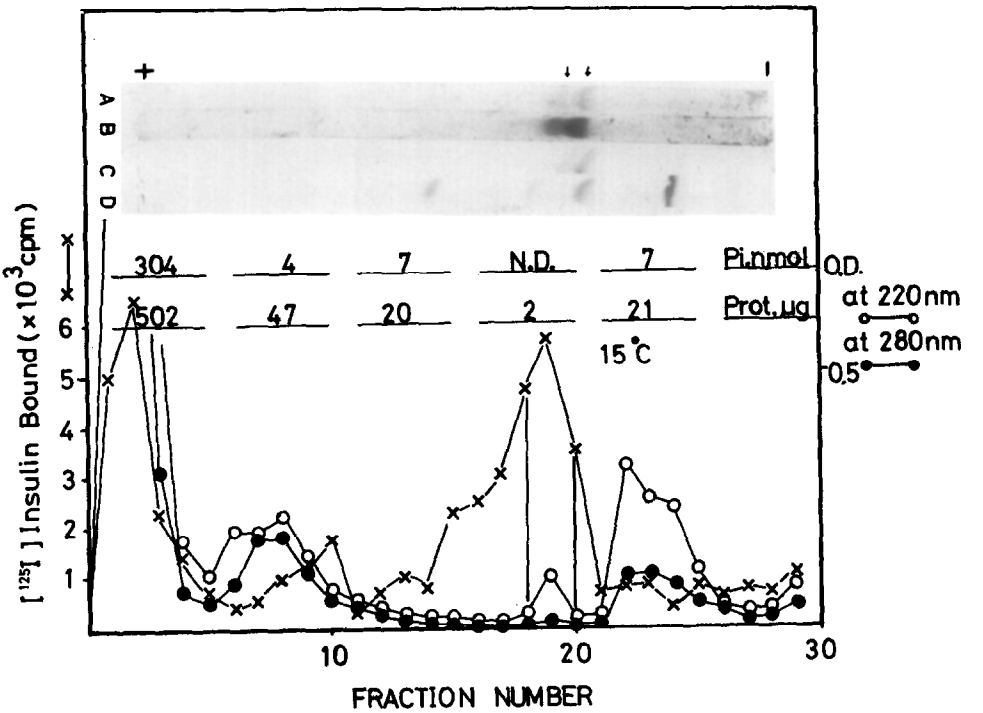

Fig. 2. Iatrobeads column chromatography of the insulin-binding substance. Elution was carried out with ethanol at 4 C (fraction 1 to 20), and then at room temperature (fraction 21 to the last). Elution was monitored by absorption at 220 nm (o—o) and 280 nm (●—●). The insulin-binding activity (×—×) of an aliquot (1 ul) of each fraction was assayed by the polyethylene glycol method as described in Methods. The concentration of ethanol in the assay mixture was 1 % (v/v). Protein (Prot.) and inorganic phosphate (Pi) were assayed as indicated in Methods.

The SDS polyacrlyamide gel electrophoresis of the purified binding protein was carried out in semimicro gradient gel (0.8 mm X 3 cm and T= 4 % to 40 %, C= 2 %)

A. binding substance from insulin-Sepharose affinity column chromatography.
B. binding substance from Concanavalin A-Sepharose column chromatography.
C. binding substance from Iatrobeads column chromatography.
D. standard proteins, (from the right), ß,ß' subunits of RNA polymerase, 165,000 and 155,000 daltons; bovine serum albumin, 68,000 daltons, subunit of RNA polymerase, 39,000 daltons; trypsin inhibitor, 21,500 daltons.

insulin-binding activity was eluted in the non-retained fractions together with most of the lipids (91 %) and proteins (72 %), but a high insulin-binding activity was also detected

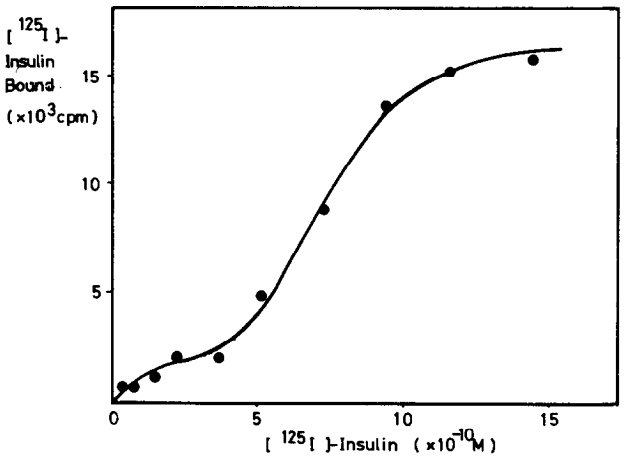

Fig. 3. Insulin-binding activity of the purified binding substance. The binding substance (about 10 ng of protein) purified by the Iatrobeads column was incubated with various concentrations of [^{125}I] insulin in the presence or absence of 50 μM native insulin after removal of ethanol from the sample by dialysis. Insulin-binding activity was assayed by the polyethylene glycol method as described in Methods. Under these conditions, nonspecific binding activity was 15-20 % of the total binding activity.

in the non-lipid containing fractions (Fig.2). An attempt was made to characterize substance present in the lipid-free fractions that were obtained.

Thereby, the substance showed reversible and specific binding activity at the physiological concentrations (10^{-11}-10^{-10}M) of insulin with binding characteristics as given in Fig. 3.[1)] In the inhibition studies, about 50 % of [^{125}I] insulin was displaced by native insulin (36 ng/ml), proinsulin and Con A (1 μg/ml), but not by glucagon and C-peptide (1 μg/ml). These data suggested that the isolated binding substance showed specific binding to insulin, and that it was a glycoprotein which could interact with Con A. Its binding activity was decreased

1) After the completion of this manuscript, Maturo III, J. M., and Hollenberg, M.D., reported a complicated biphasic binding kinetics on Triton X-100 solubilized insulin receptor, which was very similar to that of our substance as shown in Fig. 3 (21).

after the treatment with trypsin or protease free neuraminidase. 200 μg/ml trypsin abolished 50 % binding activity, and 400 μg/ml trypsin almost completely, while 400 mu/ml neuraminidase also about 50 %.[2] Therefore it was assumed that this substance was glycoprotein, even though it was relatively resistant to trypsin, and neuraminidase did not completely abolish its insulin-binding activity.

The insulin-binding substance was further purified by Con A - and insulin-affinity column chromatographies. SDS polyacrylamide gel electrophoresis on a semimicroscale of the purified insulin-binding substance showed two Coomasie Blue staining bands (Fig.2). It can not be stated with any certainty that the isolated proteins are true insulin receptors of the cell surface, in spite of their specific binding to insulin. The biphasic saturation curve is suggestive of the heterogeneity of the insulin-binding substance that was isolated.

From these observations we deduce the existence of lipophilic glycoprotein that has insulin-binding activity in rat liver.[3]

Acknowledgements

I would like to thank Dr. Noboru Yanaihara, Shizuoka college of Pharmacy, for the gift of the crystalline proinsulin used in this study. I also thank Dr. Yoshitaka Nagai, Dr, Shizuo Handa, Prof. Tamio Yamakawa for their helpful suggestions and criticism as well as stimulating discussions during this work.

---

2) This specific binding activity was also decreased by ß-galactosidase digestion (unpublished data) as reported by Caron, M. et.al. (20). But the possibility that this decrease in our experiments was caused by a possible protease contamination in the enzyme sample was not completely excluded, since protease activity was detected in a highly purified glycosidase sample except for neuraminidase (from Arthrobactor, Nakarai, Tokyo) (10).

3) It should not be overlooked, however, that a complex of glycolipid and protein might conceivably account for these results described in this paper. We could, however, not detect any glycolipids by thin layer chromatography.

References

1. Singer, S.I. (1976) Structure of Biological Membrane pp 443-462, Plenum Press, New York and London.
2. Rodbell, M. (1966) J. Biol. Chem. 241, 130-139
3. Blecher, M. (1965) Biochem. Biophys. Res. Comm. 21, 202-209.
4. Blecher, M. (1966) Biochem. Biophys. Res. Comm. 23, 68-74.
5. Cuatrecasas, P. (1971) J. Biol Chem. 246, 6532-6542.
6. Momoi, T., Ando, S., and Nagai, Y. (1976) Biochim. Biophys. Acta. 441, 488-497.
7. Cuatrecasas, P. (1972) Proc. Natl. Acad. Sci. 69, 1277-1281.
8. Batzri, S., and Korn, E.D. (1973) Biochim. Biophys. Acta. 298, 1015-1019.
9. Cuatrecasas, P. (1972) Proc. Natl. Acad. Sci. 69,
10. Uchida, Y., Tsukada, Y., and Sugimori. T. (1977) J. Biochem. 82, 1425-1433.
11. Juliano, R.L., and Stamp, D. (1976) Nature. 261, 235-238
12. Holloway, P.W. (1973) Anal. Biochem. 53, 304-308.
13. Abe, T., and Ebato, S. (1977) Seikagaku (in Japanese) 49, 1344-1347.
14. Komai, S., and Hirayuwa, N. (1969) Seikagaku (in Japanese). 41, 21-26
15. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 242. 5744-5750
16. Böhlen, P., Stein, S., Dairman, W., and Udenfried, S. (1973) Anal. Biochem. 155, 213-220.
17. Bartlett, G.R., (1959) J. Biol. Chem. 234. 466-468.
18. Cuatrecasas, P. (1973) J. Biol. Chem. 248, 3528-3534
19. Cuatrecasas, P., and Tell, G.P.E. (1973) Proc. Natl. Acad. Sci. 70, 485-489
20. Caron, M., Picard, J., and Kern. (1978) Biochim. Biophys. Acta. 512, 29-40.
21. Maturo III, J.M., and Hollenberg, M.D. (1978) Proc. Natl. Acad. Sci. 75, 3070-3074.