CHARACTERIZATION OF AN [^{125}I]-INSULIN BINDING PLASMA MEMBRANE FRACTION FROM RAT LIVER

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Summary

A procedure for the isolation of three different plasma membrane fractions from rat liver is described. The heavy subfraction (PL_1) has a high specific activity in alkaline phosphatase, while the lightest subfraction (PL_3) contains a high specific activity in 5' -Nucleotidase, ATPase and phosphodiesterase. The major part of the plasma membrane recovered is found in PL_3. A specific binding of [^{125}I]-Insulin is shown to occur in PL_3, with a ten-fold enhancement over that of any other plasma membrane fraction.

The action of insulin at the plasma membrane surface requires at least one, if not two, specific receptor sites. It has been suggested that the interaction of insulin with the plasma membrane results in a physical change in the structure of the membrane (1). The main effects of insulin on glycolysis, gluconeogenesis and protein metabolism in the hepatic cell are possibly brought about by lowering the intracellular cyclic AMP levels (2,3). However, it has been shown that the effect of insulin on glucose transport is not related to the intracellular level of cyclic AMP (4).

Any attempted analysis of the insulin receptor site(s) requires a careful preliminary characterization of the various membrane fragments. In the present work we have tried, on the basis of an enzymatic analysis of plasma membrane fragments, to correlate recognizable membrane parameters with insulin binding capacity in three different membrane fractions from rat liver.

Methods

Rat liver plasma membranes were prepared by a modification of the methods of Coleman et al (5) and Wallach and Kamat (6). Male rats (200-250g) were starved for 18 hours and the livers removed after stunning. A Teflon-glass homogenizer (radial clearance 0.005-0.007 in) was used to hand-homogenize the minced livers (7-8 up and down strokes) in 0.25M sucrose and 5mM Tris-HCl, pH 7.4 (Medium H). The homogenate was adjusted to 10ml/g liver after filtration.

The filtered homogenate was spun for 10 mins at 350xg, and the pellet was washed once after rehomogenization. The combined supernatants were centrifuged at 1,500xg for 10 mins, and the pellet material was loaded onto discontinuous sucrose gradients of the following densities: 1.30-5ml; 1.21-5ml; 1.195-5ml; 1.184-10ml; 1.171-10ml and 1.157-10ml. These were centrifuged at 25,000 RPM for 90 mins (Spinco SW 25.2) and the fractions were collected, washed and centrifuged in Medium H at 10,000xg for 10 mins. A second discontinuous gradient similar to the first was carried out and the collected fractions were stored at -20°C until assayed.

The supernatants obtained from the 1,500xg spin were centrifuged for 10 mins at 10,000xg, and the pellet was designated the mitochondrial fraction. The supernatant was centrifuged at 75,000xg for 1 hour. The pellet was taken up into medium H and layered onto a Ficoll gradient of the following densities: 1.104-5ml; 1.055-10ml and 1.036-10ml. The gradients were spun at 25,000 RPM for 90 mins. (Spinco SW 25.1) and the collected fractions were washed in Medium H and stored at -20°C.

Results

In the three plasma membrane fractions (Table 1) (PL_1-1.18

TABLE I

Enzyme Activities of Plasma Membrane Fractions and Other Subcellular Fractions.

Enzyme	Homog.	PL_1	PL_2	PL_3	SER	RER	Mito	Sol.
5' -Nase (6)	1.48	1.79	11.8	32.6	6.0	1.6	--	--
Alkaline-Pase (6)	0.07	1.46	0.46	0.15	0.06	0.06	--	--
PDase (6)	0.07	0.18	0.22	0.31	0.15	--	--	0.07
$(Na^+ + K^+)$-Mg^{2+} ATPase (3)	1.64	2.15	14.1	14.5	1.56	1.15	--	--
SDH (3)	2.26	3.10	0.91	0	--	--	20.7	--
G-6-Pase (3)	2.95	0.16	0.76	0.34	--	11.7	--	--
Yield of protein (mg/g liver)	189	0.60	0.39	2.61				

Results are expressed in μmoles product per mg protein per hour. All results in each fraction agree to within 20% in all cases. Figures in parenthesis represent the number of preparations. The specific activities are an average of the total number of preparations. Homog. - Homogenate; PL - Plasma membrane; SER - Smooth endoplasmic reticulum; RER - Rough endoplasmic reticulum; Mito. - Mitochondria and Sol. - Soluble fraction. The following enzyme assays were used: 5' -Nucleotidase (5' -Nase) (13); Alkaline phosphatase (Alkaline-Pase) (14); Phosphodiesterase (PDase) (14); ATPase (15); Succinate dehydrogenase (SDH) (16) and Glucose-6-Phosphatase (G-6-Pase) (17). Liberated Pi was determined by the method of Ames (18), protein by the method of Lowry et al (19).

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sucrose density; PL_2-1.17 sucrose density and PL_3-1.036 Ficoll density), 5' -Nase, PDase and ATPase were found at high specific activity in PL_3 and to a lesser extent, in PL_2. In fact, the relative specific activity (RSA) of 5' -Nase activity in PL_3 was more than 20 when compared with the homogenate. This represents more than 30% of the total 5' -Nase activity found in the homogen-

ate. Fraction PL_1 contained very little of the above mentioned enzymes but did appear to be very active in Alkaline-Pase (RSA = 22.4).

Contamination of the plasma membrane fractions was determined from the activities of mitochondrial (SDH) and microsomal (G-6-Pase) enzymes. On this basis PL_1 contained approximately 15% mitochondria, PL_2 less than 5% and PL_3 had none. Microsomal contamination was 6.5% in PL_2, 2.9% in PL_3 and 1.4% in PL_1. It should be noted that the SER fraction contained more plasma membrane contamination, as evidenced by 5' -Nase activity, than PL_3 contained microsomal contamination.

Preliminary experiments aimed at determining insulin binding

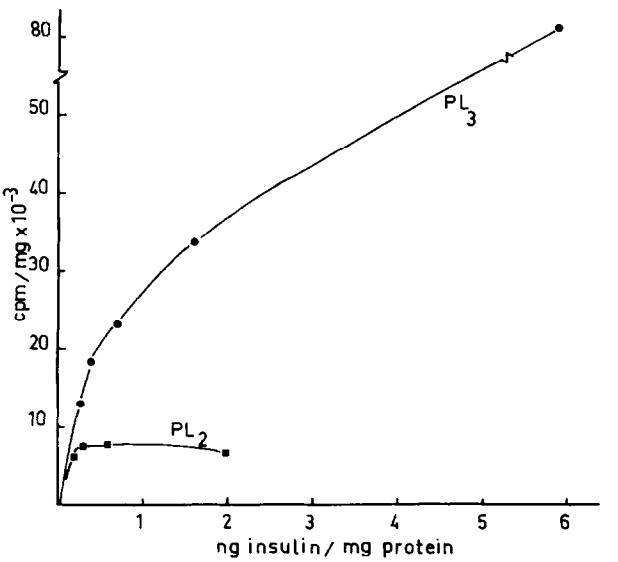

Figure 1.

Insulin binding is expressed as cpm/mg protein. The preparations were kept at -20°C for 1 week. Insulin binding was determined with a 50µl aliquot of each fraction in the presence of 0.5ml Krebs-Ringer phosphate buffer, pH 7.4, incubated at 37°C for 10 mins. [^{125}I] -insulin (50-100µC/µg insulin) was used and the reaction was stopped with 0.5ml ice-cold 10% TCA. The tubes were centrifuged for 5 mins and the free insulin was extracted with acidic n-butanol. The pellet was homogenized and suspended in 15ml scintillation cocktail. Less than 8% of the [^{125}I] -insulin used was soluble in 5% TCA and this is accounted for in the results.

parameters have shown that fraction PL_3 has a much higher capacity to bind insulin than fraction PL_2, (Fig. 1). The difference in the amount of $[^{125}I]$-insulin* bound can be as great as 10-fold among the various membrane fractions and more than 20-fold greater than the homogenate. The amount of insulin binding/mg protein also increased with a decrease in the density of the plasma membrane fraction, as did the SH-group content/mg (not shown).

It was observed that the binding was resistant to freezing (-20°C) for up to one week. It has also been noted that maximal binding occurs in less than 2.5 mins at 0°C, from which it might be expected that at 37°C the binding is completed even more quickly. This indicated that the time allowed for the binding studies was sufficient to observe a maximal response.

Discussion

The results show that the rat liver plasma membrane is composed of at least three membrane fractions of different densities and enzymatic activities. Evans (7) has presented similar results obtained with a zonal centrifugation technique, though it must be pointed out that he obtained his light and heavy subfractions by rehomogenization of an intermediate fraction (sucrose density 1.17). In this paper it has been shown that the three subfractions of the plasma membrane have been obtained following a single initial homogenization, suggesting a less fragmented origin for these fractions.

By the use of widely differing methods several investigators have isolated plasma membrane fractions with densities of 1.13 (5), 1.16-1.19 (8) and 1.16 and 1.18 (9). In none of these cases have

* (^{125}I - Insulin (Lot 77) was purchased from the Radiochemical Center, Amersham. The purity of this compound was checked by Sephadex chromatography and TCA precipitation.

Table 1. The effect of age on carboxylation of pyruvate by human and rat adipose tissue mitochondria.

The reaction mixture was as described in Figure 1. Incubations were carried out at 37° for 30 minutes.

Source	Age	Pyruvate carboxylation
		mμmoles $H^{14}CO_3^-$ fixed/mg protein
Human	22 years	39
	54 years	37
Rat	6 weeks	1798
	6 weeks	1895
	10 weeks	350
	14 weeks	238

view of this a possible effect of aging on adipose tissue pyruvate carboxylation was investigated. As seen in Table 1 mitochondrial carboxylation of pyruvate decreases markedly in adipose tissue with increasing age of the rat. A similar magnitude of reduction in lipogenesis in rat adipose tissue from older animals has been observed (14). In contrast to the aging phenomenon observed in rat adipose tissue the carboxylation of pyruvate by human adipose tissue mitochondria (Table 1) is not affected by age.

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spite of the readily demonstrable effect of insulin on glucose transport in adipose and muscle tissue, its effect on hepatic metabolism is possibly one of the most important aspects of its action.

These observations strongly suggest that the origin of the light subfraction (PL_3) arises from the sinusoidal surface of the parenchymal cells. This surface would be appropriate for the action of insulin in affecting hepatic metabolism. A further examination of this problem is being carried out on the localization of adenyl cyclase. As this enzyme has been shown (12) to reside in at least two different morphological sites in hepatic tissue, its positive identification in the present fractions may lead to the resolution of the source of the various plasma membrane fractions in liver tissue.

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