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INSULIN INHIBITS NADH-SEMIDEHYDROASCORBATE REDUCTASE IN RAT LIVER PLASMA MEMBRANE

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SUMMARY:

Plasma membrane fractions from rat liver isolated by different methods contain NADH- ferricyanide reductase and NADH-semidehydroascorbate reductase activities which are too high to be due to contamination by mitochondria or microsomes. Both enzymes are inhibited by insulin in a concentration range of 30 to 70 μU insulin /ml down to 20% residual activity. While ferricyanide reductase returns to near normal activity, semidehydroascorbate reductase inhibition persists at higher insulin concentrations. Despite variations in basal enzyme activities, degree of inhibition and amount of hormone needed for maximal effect, it can be consistently observed. Inhibition of semidehydroascorbate reductase is discussed in terms of the enzyme's function in regenerating ascorbate as antioxidant and oxidant-like insulin effects.

It has been reported earlier that certain hormones influence redox function in plasma membranes (1-5). In rat and mouse liver as well as fat cell membranes NADH-indophenol reductase is partly inhibited by insulin in physiological concentrations. Under appropriate conditions this effect can also be shown with ferricyanide or cytochrome c, but not molecular oxygen, which is relatively rapidly reduced by NADH in liver plasma membranes (4,6,7). Since the dyes used including cytochrome c are all artificial electron acceptors the question arises as to whether the reduction of a physiological acceptor is also influenced by the hormone.

We have found significant activities of NADH-semidehydro-ascorbate reductase (E.C. 1.6.5.4.)(8) in rat liver plasma membranes obtained by two different methods (9,10) and investigated the effect of insulin on this enzyme which is important for the regeneration of ascorbate, one of the most powerful radical scavengers in the cell.

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Inhibition of this enzyme may lead to at least local accumulation of free radicals in the vicinity of the membrane and according secondary effects, like, i.e., oxidation of sulfhydryl groups in proteins with subsequent activation or deactivation of enzymes or transport molecules. A mechanism of this type for insulin action has been proposed for fat cells (5,11,12).

METHODS :

Plasma membranes from rat liver were isolated by the methods of Yunghans and Morré (9), with 0.5 mM $CaCl_2$ in the homogenization medium (13)("nuclear" plasma membrane) and Touster & al (10)("microsomal" plasma membrane), respectively. The 100 000xg pellet from the latter fractionation was used as reference microsomal fraction. A mitochondrial fraction was prepared as described by Morré (14). Fraction purity was estimated by assay of appropriate marker enzymes, 5'-nucleotidase (15) and alkaline phosphodiesterase (10) for plasma membrane, glucose-6-phosphatase (16) for endoplasmic reticulum and succinate-INT§-redutase (17) for mitochondria. Dehydroascorbic acid was purified by recrystallization of commercially available material from glacial acetic acid-HCl (18), and NADH-semidehydroascorbate reductase was assayed spectrophotometrically at 340 nm as described by Lumper & al (19) in 50 mM Tris-HCl, pH 7.5. NADH-ferricyanide reductase was assayed under the same conditions with 0.2 mM $K_3(Fe(CN)_6)$ and 80 µM NADH in a total volume of 1 ml. Insulin (porcine, 24.1 U/mg, from Lilly Corp.) was diluted in buffer containing 1% bovine serum albumin and either added to the assay cuvette together with the sample or preincubated with the membrane in 10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and a 100 µl aliquot added to the assay mixture. Protein was determined by the Bio-Rad dye binding assay (20).

RESULTS :

It can be seen from the data in table 1 that plasma membrane fractions obtained by either method contain NADH-dehydrogenase and NADH-semidehydroascorbate reductase activities not accountable for by microsomal or mitochondrial contamination. The plasma membrane origin of the dehydrogenase activity is further substantiated by its relative resistance to inhibition by p-chloromercuribenzoate (75% and 85% inhibition, respectively, in "nuclear" and "microsomal" plasma membrane, in contrast to 97% for microsomal dehydrogenase) (7).

§) Abbreviation: INT, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride.

TABLE I

SPECIFIC ACTIVITIES AND RELATIVE SPECIFIC ACTIVITIES OF MARKER AND REDOX ENZYMES IN RAT LIVER CELL FRACTIONS.

ENZYME	"NUCLEAR" PLASMA MEMBRANE	"MICROSOMAL" PLASMA MEMBRANE	MICROSOMES	MITOCHONDRIA
5'-Nucleotidase				
Specific Activity[a]	1900	1250	370	n.m.[b]
Relative Specific Activity[c]	40	28	8.6	
Alkaline Phosphodiesterase				
Specific Activity	n.m.	92	9.1	n.m.
Relative Specific Activity		28	2.7	
Glucose-6-Phosphatase				
Specific Activity	100	115	916	n.m.
Relative Specific Activity	0.76	0.8	6.4	
Succinate-INT-Reductase				
Specific Activity	27	n.d.[d]	116	424
Relative Specific Activity	0.6		2.7	9.8
NADH-Ferricyanide-Reductase				
Specific Activity	1500	2300	3500	3800
NADH-Semidehydroascorbate-Reductase				
Specific Activity	20	16.9	33.5	5.2

a) Specific activities are expressed as nmoles of product formed or substrate consumed per minute . mg of protein
b) n.m., not measured.
c) Relative specific activity, recovery of enzyme activity in the fraction / recovery of protein in the fraction.
d) n.d., not detectable with this assay, specific activity below 0.2 nmoles / min.mg protein.

Both enzyme activities in both fractions are partly inhibited by insulin at concentrations between 30 and 60 μU/ml. If the insulin concentration is raised (above 60-90 μU/ml), ferricyanide reductase activity rises whereas semidehydroascorbate reductase remains at low levels (Fig. 1). We also observed complete inhibition in some experiments above 60 μU/ml, but this is not always reproducible. In general, the inhibition can be shown most reliably if the membrane is preincubated with insulin for 5 to 10 minutes at room temperature (see Methods). The data shown are each from one experiment, because the amount of insulin varies as well as the degree of inhibition, but the effect itself is consistent.
In mitochondrial and microsomal fractions NADH-semidehydroascorbate reductase is inhibited only 10-15%, NADH-ferricyanide reductase is not inhibited at all by insulin.

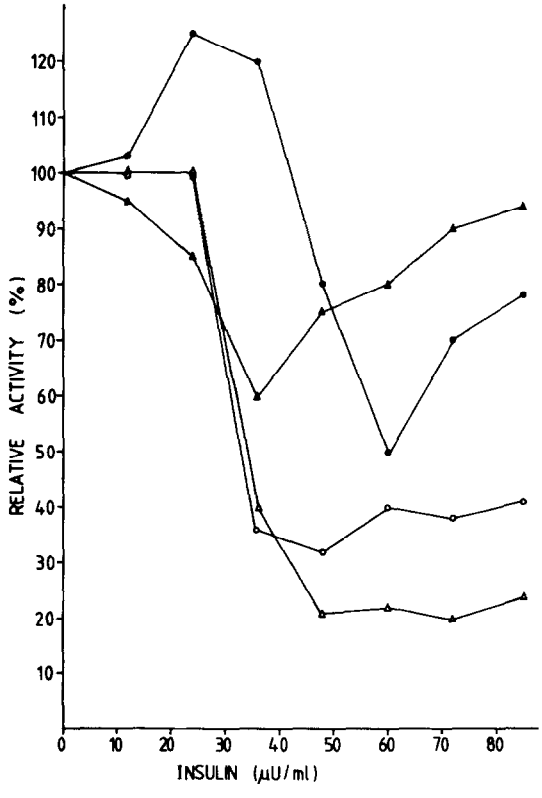

Figure 1
Influence of insulin on NADH-ferricyanide reductase (closed symbols) and NADH-semidehydroascorbate reductase (open symbols) in rat liver plasma membrane isolated according to Yunghans & Morré (9) (circles) and Touster & al (10) (triangles). Data obtained after preincubation of membrane samples with insulin at pH 8.0, assays at pH 7.5.

DISCUSSION :

The molecular mechanism of insulin action is virtually unknown apart from the certain fact that it binds to the cell membrane (21). Recently, based on experimental evidence obtained in fat cells, two kinds of suggestions have been made. One is direct influence of insulin on ATPase activity and phosphorylation of membrane proteins and subsequently mitochondrial pyruvate dehydrogenase (22), the other is enzymatic generation of H_2O_2 which can act as a second messenger of insulin action (5,12). In respect to the latter hypothesis it is interesting that insulin inhibits NADH-dehydrogenase in liver and fat cell membranes (2,3) as well as erythrocyte membranes (23), but this has been discussed as influence on energy dependent proton pumping in the membrane rather than generation of a molecular second messenger.
We have found inhibition of NADH-semidehydroascorbate reductase in liver plasma membrane, an enzyme which presumably acts in regeneration of reduced ascorbate thereby restoring its antioxidant function (8). The bulk of this enzyme in liver is probably not localized in hepatocytes but in membranes of other cell types (e.g., Kupffer cells, which have a strong peroxide metabolism) (24), but a role of this enzyme in protection of microsomal and outer mitochondrial membranes from lipid peroxidation has been suggested (25). Inhibition of NADH-semidehydroascorbate reductase in plasma membranes may lead to an increase in the concentration of free radicals which may exert oxidative effects observed in insulin action (11).
Although the two different plasma membrane fractions are described as being largely derived from different parts of the hepatocyte surface having different concentrations of hormone sensitive adenylate cyclase (26), we find no significant difference in the observed insulin effect, which might be expected to be found mainly in the sinusoidal front membrane, corresponding to the "microsomal" plasma membrane fraction. This, of course, does not exclude polarity concerning the physiological significance of our observation.

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