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Insulin effect in vitro on human erythrocyte plasma membrane[1]

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Summary. The effect of porcine insulin has been tested in vitro on human erythrocyte plasma membrane (Na^+-K^+) and Mg^{2+}-ATPase activities as well as on membrane fluidity. The results indicate that the hormonal treatment significantly inhibits (Na^+-K^+)-ATPase activity, and at the same time decreases membrane fluidity.

The presence of a specific insulin receptor on the human erythrocyte plasma membrane has recently been reported[2]. The physiological relevance of this observation is not yet clear, even if altered binding capacities observed in red cells from diabetic patients could be related in some way to metabolic defects[3]. The effect of insulin on isolated mammalian plasma membranes in relation to membrane-bound enzymes has been reported rather extensively[4-9], and very recently a direct action of the hormone on the liver plasma membrane microenvironment has been shown[10,11]. This body of evidences prompted us to study the effect of insulin in vitro on human erythrocyte membrane-bound Mg^{2+}-ATPase (E.C.3.6.1.3) and (Na^+-K^+)-ATPase (E.C.3.6.1.4) activities as well as a possible hormonal action on plasma membrane fluidity.

Materials and methods. Human erythrocyte plasma membranes were prepared from fresh blood, obtained from healthy adult male volunteers, according to Hanahan[12].

Mg^{2+}-ATPase and (Na^+-K^+)-ATPase activities were measured as previously reported[7] in a final volume of 2.2 ml containing the following; about 100 µg membrane proteins, 92 mM Tris-HCl buffer (pH 7.5), 5 mM $MgSO_4$, 5 mM KCl, 60 mM NaCl, 0.1 mM EDTA and 0.1 mM ouabain when employed; ATP concentration was 4 mM.

Fluorescence labeling of human erythrocyte plasma membranes was carried out as follows; $2 \cdot 10^{-3}$M 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofuran was diluted 1:1000 just before use with the hypotonic Tris buffer used during membrane isolation, then mixed in a 1:1 ratio with the membrane suspension to give a final protein concentration of 50 µg/ml. After 15 min of incubation at 37 °C fluorescence polarization measurements were carried out as recently reported[13] with an Aminco Bowman spectrophotofluorometer equipped with 2 Glan prism polarizers; excitation was set at 366 nm and emission was recorded at 430 nm. The temperature of the sample was checked within ±0.1 °C with a thermistor thermometer.

The degree of fluorescence polarization, P, was calculated from the equation:

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} = \frac{(I_\parallel / I_\perp) - 1}{(I_\parallel / I_\perp) + 1}$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities recorded with the analyzing polarizer oriented, respectively, parallel and perpendicular to the direction of the polarized excitation beam[14].

Porcine insulin was denatured according to Loten and Sneyd[4]. Proteins were estimated by the method of Lowry et al.[15] using bovine serum albumin as a standard. Porcine insulin was from Sigma, St.Louis, Mo., USA; porcine proinsulin was obtained from Lilly Research Laboratories, Indianapolis, Ind. USA; ATP was from Boehringer, Mannheim, FRG; 1,6-diphenyl-1,3,5-hexatriene was from Fluka AG, Buchs, Switzerland; all other chemicals were analytical reagent grade from Merck-Darmstadt, FRG.

Table 1. Insulin effect on (Na^+-K^+)-ATPase, Mg^{2+}-ATPase and fluorescence polarization (P at 37 °C) of human erythrocyte plasma membrane

Preincubation time (min)	(Na^+-K^+)-ATPase		Mg^{2+}-ATPase		Fluorescence polarization (P)	
	− Insulin	+ Insulin	− Insulin	+ Insulin	− Insulin	+ Insulin
0	0.62±0.15	0.60±0.16	3.20±0.30	3.40±0.40	0.268±0.008	0.270±0.006
30	0.58±0.12	0.40±0.07[a,b]	3.25±0.28	3.25±0.24	0.269±0.007	0.286±0.004[a,b]
60	0.55±0.14	0.35±0.05[a,b]	3.15±0.23	3.30±0.25	0.272±0.006	0.289±0.005[a,b]
120	0.50±0.10	0.27±0.06[a,b]	3.00±0.19	3.19±0.18	0.270±0.009	0.290±0.007[a,b]
180	0.57±0.11	0.25±0.04[a,b]	2.95±0.16	3.14±0.20	0.274±0.005	0.294±0.006[a,b]

Erythrocyte membranes were preincubated at 37 °C for the time indicated in the presence or absence of $1 \cdot 10^{-9}$ M porcine insulin, before starting the reaction by adding the substrate or measuring the P value. Enzyme activity is reported as µmoles P_i/mg protein per 5 min; results are means ±SD of 6 different membrane preparations. $p < 0.05$, at least, [a] with respect to untreated controls, or [b] with respect to zero time, as assessed by Student's t-test.

Results and discussion. Insulin treatment in vitro inhibits (Na^+-K^+)-ATPase activity of human erythrocyte plasma membrane, this effect being dependent on the length of preincubation in the presence of the hormone; Mg^{2+}-ATPase shows no significant sensitivity to the hormonal treatment (table 1). The degree of fluorescence polarization, P, increases with time on insulin treatment (table 1) thus suggesting a decrease in the lipid fluidity of the plasma membrane microenvironment[14,16]. The insulin effect on both (Na^+-K^+)-ATPase activity and fluorescence polarization, P, appears to be significant after 30 min of preincubation of plasma membranes and remains fairly constant up to 3 h. Such a stable effect could be ascribed to the very much reduced dissociation from membrane receptors and to the negligible degradation observed for insulin interaction with human erythrocyte plasma membranes as compared with other similar mammalian systems[2], even if its reversibility was not investigated. In addition, it is also interesting to remark that if on one hand the total ATPase activity (i.e. (Na^+-K^+)-ATPase plus Mg^{2+}-ATPase) remains unchanged after extensive preincubation and the same is true for the ouabain-sensitive fraction - namely the (Na^+-K^+)-ATPase - which covers about 16% of total activity. On the other hand the insulin treatment leaves the total activity unaltered, decreasing the ouabain-sensitive fraction to about 7% of total activity (table 1); an analysis of variance showed the significance ($p<0.05$) of the reported sensitivity of (Na^+-K^+)-ATPase activity to insulin treatment in vitro for different time intervals.

A dose-dependent study was carried out on the sensitivity to the in vitro insulin treatment of both ATPase activities and fluorescence polarization of erythrocyte plasma membranes. Results reported in table 2 show the complete insensitivity of Mg^{2+}-ATPase to the hormonal treatment in vitro in the concentration range tested. (Na^+-K^+)-ATPase activity is significantly inhibited at concentrations as low as $5 \cdot 10^{-10}$M (not shown); in addition the fraction of total activity represented by the (Na^+-K^+)-ATPase is decreased, after insulin treatment as reported in table 2, from about 17% to less than 10%; such a variation, observed throughout the hormone concentration range, is significant as assessed by an analysis of variance ($p<0.05$). Fluorescence polarization values are significantly increased over a wide range of hormone concentrations, thus indicating a concentration-dependence of the stiffening effect elicited by the hormonal treatment in vitro on the human erythrocyte membrane microenvironment.

The Hill coefficient n[17] calculated for (Na^+-K^+)-ATPase of human erythrocyte membrane from equation log [v/(Vmax − v)] = n log(S)-log K in a Na^+ concentration range 0–10 mM was 1.62 ± 0.32 and 1.27 ± 0.21 (mean ± SD; n = 8) respectively for membranes preincubated for 30 min at 37 °C without and with $1 \cdot 10^{-9}$M porcine insulin before the addition of the substrate. The reported difference is significant ($p<0.02$) as from paired data analysis; such a result suggests that the positive cooperative behaviour of the enzyme[18] is negatively affected by the in vitro hormonal treatment.

In order to assess the specificity of the insulin effect, the experiments reported so far were carried out also in the presence of both porcine proinsulin and denatured porcine insulin; results, which are not reported here for the sake of brevity, did not indicate any statistically significant effect.

The hormonal effect on the erythrocyte membrane microenvironment was further investigated on membranes treated as reported in table 2 with or without $1 \cdot 10^{-9}$ M insulin; the flow activation energy ΔE was determined from the empirical relation[16] $\eta = Ae^{\Delta E/RT}$ as previously reported[13]. The values observed for ΔE were 8.0 and 7.6 kcal · mol^{-1} for untreated and insulin-treated erythrocyte membranes respectively; this difference, which is significant ($p<0.01$) as from the analysis of variance of experimental results (n = 7), is suggestive of an increasing order[14,16] of the membrane structure due to the insulin treatment, even though one should always take into account the questioned validity of the steady-state fluorescence polarization technique as related to the possible microheterogeneity of the fluorophore solvation sites in the membrane interior[19]. The role of membrane fluidity with reference to the activity of membrane-bound enzymes has received increased attention in recent years[20,21] and, in particular, the decrease in membrane fluidity has been frequently associated with a decrease in both (Na^+-K^+)- and Ca^{2+}-ATPase activities[22-24]. In the same line, experimental observations relating hormonal binding to membrane fluidity can be considered[10,25]. In this respect, even if the physiological significance of the presence of insulin receptors on human erythrocyte plasma membrane is still to be elucidated[3], our results suggest that the in vitro interaction of the hormone with its receptor could negatively affect membrane fluidity; this alteration of the membrane microenvironment might be responsible for the decrease of (Na^+-K^+)-ATPase activity, an effect which could in turn be related to the altered cooperative behaviour of the enzyme[18]. In this context, it has recently been reported that an increased fluidity stimulates sodium pumping activity in human erythrocytes[26]. It should also be recalled that altered rheological properties, ascribed at least partially to changes of plasma membrane viscous properties, have been reported in erythrocytes from human diabetic patients[27], and these observations have been related to a derangement of the plasma membrane microenvironment[28].

Table 2. Dose-response study of the insulin effect on (Na^+-K^+)-ATPase, Mg^{2+}-ATPase and fluorescence polarization (P at 37 °C) of human erythrocyte plasma membrane

Insulin (M)	(Na^+-K^+)-ATPase	Mg^{2+}-ATPase	Fluorescence polarization (P)
0	0.65 ± 0.20	3.15 ± 0.25	0.270 ± 0.005
10^{-11}	0.62 ± 0.24	3.20 ± 0.30	0.274 ± 0.004
10^{-10}	0.54 ± 0.15	3.10 ± 0.60	0.280 ± 0.006^a
10^{-9}	0.40 ± 0.07^a	3.40 ± 0.65	0.288 ± 0.007^a
10^{-8}	0.35 ± 0.05^a	3.35 ± 0.35	0.292 ± 0.004^a

Erythrocyte membranes were incubated for 30 min at 37 °C with the indicated concentration of porcine insulin before starting the reaction by adding the substrate or measuring the P value. Enzymatic activity is reported as μmoles P_i/mg protein per 5 min; results are means ± SD of 6 different membrane preparations. [a] $p<0.05$, at least, with respect to untreated controls as assessed by Student's t-test.

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In vivo effect of inhibin on FSH uptake by rat testis

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Summary. Simultaneous administration of inhibin and ^{125}I-hFSH significantly decreased the uptake of ^{125}I-hFSH by the immature rat testis. The data suggest that inhibin may modulate FSH action at the gonadal level.

It has now been well established that a gonadal factor, inhibin is involved in the feedback control of FSH secretion. Inhibin acts on the pituitary both in vitro and in vivo as shown by alterations observed in responsiveness to LH-RH[1-4]. However, recent evidence based on in vitro studies suggests that inhibin affects the sensitivity of the testicular tissue to LH as well as FSH[5-8]. Such evidence implies that inhibin may have multiple sites of action. In the present study, we have examined the in vivo effect of inhibin on the uptake of ^{125}I-hFSH by immature rat testis.

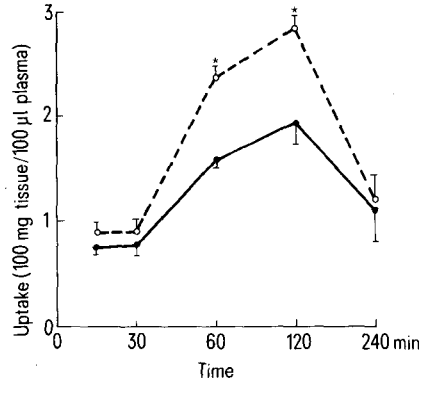
Materials and methods. A homogeneous preparation of inhibin (hSPI) isolated from human seminal plasma, having a mol.wt of 19,000 daltons, was used in the present study[9]. hFSH (NIN-FSH HS-1) kindly supplied by NIAMDD-Bethesda was used as a radioligand. This preparation had a FSH biological activity, as measured in the hCG augmented ovarian weight gain assay[10] of 4990 IU/mg and LH activity as 183 IU/mg as measured by the ovarian ascorbic acid depletion assay[11]. The same preparation was iodinated using the chloramine T method with a sp.act. of 72 μCi/μg. 27-day-old male rats of the Holtzman strain were used. The control animals were injected with single i.v. injection of [^{125}I-hFSH (10 ng) + BSA (50 μg)]/100 μl and experimental animals with [^{125}I-hFSH (10 ng) + hSPI (50 μg)]/100 μl in PBS pH(7.0) respectively via the femoral vein. In both groups animals were bled via heart puncture at different time intervals. Testes were removed, washed with saline and weighed, and the total radioactivity was measured in a γ-ray spectrometer. Radioactivity was expressed as cpm per mg weight or per ml plasma. Uptake was calculated as the ratio of cpm per g weight of tissue/cpm per ml plasma.

Results. Maximum uptake (tissue/plasma) of ^{125}I-hFSH by immature rat testis was 2.8 at 120 min in control rats. Thereafter the uptake of labelled hormone by the testis declined and reached a nadir by 6 h. In the experimental group, simultaneous administration of ^{125}I-hFSH along with inhibin suppressed the uptake of FSH to 1.9 at 120 min (figure). This effect of inhibin on FSH uptake was observed throughout the time interval studied.

In a separate experiment, it was observed that simultaneous injection of 2 μg of unlabelled hFSH via the i.p. route

Specificity of ^{125}I-hFSH uptake by immature rat testis, observation made at 120 min

	Counts per testis ^{125}I-hFSH (10 ng)	^{125}I-hFSH + hFSH (10 ng + 2 μg)
1	4280	751
2	3926	976
3	3776	1121
4	3996	851



Uptake of radioactivity by the testis in control (○---○) and experimental (●——●) animals. The decrease in FSH uptake at 60 min and 120 min was significant, $p < 0.02$ and $p < 0.001$ respectively (Student's t-test). Plotted values represent the mean ± SE for 4 animals.