Covalent glycoinositolphospholipid (GPI) binding to hemoglobin is associated with insulin-activation of erythrocyte membrane protease

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Abstract: Recently, it was demonstrated that prolonged hyperinsulinism associated with hypoglycemia, both *in vivo* and *in vitro*, caused covalent glycoinositolphospholipid (GPI) binding to the C termini of both hemoglobin β-chains, which resulted in the formation of a novel, hitherto unrecognized, minor hemoglobin fraction (GPI-Hb) (Niketić *et al.*, Biochem. Biophys. Res. Commun. **239** (1997) 435). In this study it was demonstrated that exposure of erythrocyte membranes to insulin causes the activation of membrane protease as well as that the formation of GPI-Hb parallels its activity. It is suggested that the insulin-activated protease is able to catalyze, albeit slowly, the transpeptidation, *i.e.*, the replacement of the carboxy-terminal amino acid(s) residues of the Hb β-chains with GPI as an exogenous nucleophile. To our knowledge the present results show for the first time that insulin stimulates protease activity in erythrocyte membranes, as well as that insulin-activated protease may be involved in post-translational GPI binding to proteins.

Keywords: insulin, hemoglobin, glycoinositolphospholipid, erythrocytes, protease.

INTRODUCTION

Human erythrocytes contain highly specific insulin receptors that have structural and functional characteristics similar to those of target tissues for the hormone action.[1,2] We demonstrated recently that prolonged hyperinsulinism associated with hypoglycemia, both *in vivo* and *in vitro*, caused covalent glycoinositolphospholipid (GPI) binding to C termini of both hemoglobin β-chains, which resulted in the formation of a novel, hitherto unrecognised, minor Hb fraction (GPI-Hb).[3] Free GPI-lipids from human erythrocyte membrane, as opposed to other cell types, are largely cytoplasmatically oriented and insensitive to insulin-induced hydrolysis.[4,5] Binding of insulin to its receptor in tar-

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get cells stimulates a membrane protease,[6] and this enzyme was suggested to promote hydrolysis of GPIs from the GPI-anchors of GPI-proteins.[7] We hypothesized that the molecular mechanism by which insulin induces GPI-transfer to Hb involves activation of red cell membrane protease, which is able to catalyze transpeptidation, *i.e.*, the replacement of the carboxy-terminal amino acid(s) residues of Hb β-chains with GPI as an exogenous nucleophile.[3] The present study was undertaken to test the proposed hypothesis. The results demonstrate that exposure of erythrocyte membranes to insulin causes the activation of a membrane protease with a trypsin-like specificity, as well as that the formation of GPI-Hb parallels its activity. It is suggested that the insulin-activated protease is able to catalyze, albeit slowly, transpeptidation, *i.e.*, the cleavage of peptide bond between Lys-144 and Trp-145 of the Hb β-chain followed with replacement of Trp residue with GPI as an exogenous nucleophile. To our knowledge the present results show for the first time that insulin stimulates protease activity in erythrocyte membranes and demonstrates that this protease may be involved in the transfer of GPI-lipid to the protein in response to insulin binding to the cell.

EXPERIMENTAL

Fresh blood samples (2–5 mL) were taken from normal healthy volunteers and the erythrocytes were separated as described previously.[8] Hemolysis of the erythrocytes and isolation of the membranes were performed as described by Kundu *et al.*[9] Membrane protein (MP) concentrations were measured using a modified Lowry procedure.[10] The major hemoglobin, the HbAo fraction, was isolated from the hemolysates by ion exchange chromatography on a Bio-Rex column[8] followed by concentration by ultrafiltration and extensive dialysis against 60 mM sodium phosphate buffer used for the incubation experiments. ATP, BAPNA (N_α-benzoyl-DL-arginine *p*-nitroanilide), BAME (N_α-benzoyl-L-arginine methyl ester) and TLCK (N_α-*p*-tosyl-L-lysine chloromethyl ketone) were all from Sigma. Membranes (5 mg MP/mL) were suspended in 60 mM sodium phosphate buffer pH 7.4 containing 10 μM ATP and graded concentrations of TLCK. Incubation with 1 μg/mL of insulin (NOVO, Denmark) in the presence and absence of HbAo (30 mg/mL) was done at 37 ºC with agitation. Controls without insulin were run in parallel. After incubation, the aliquots were removed at various times for measurement of the membrane protease activity and estimation of GPI-Hb. The membranes were sedimented at 22000 g for 15 min at 4 ºC and GPI-Hb was estimated in the supernatant by applying a locally developed mini column method.[11] For the protease activity measurements, the membranes were washed three times in the same buffer (in which TLCK had been added to the concentration corresponding to that in the incubation mixture) to remove insulin and then, resuspended in 1 mL of the same buffer. Protease activity was estimated by applying the chromogenic substrate BAPNA as described by Cohen.[12] The results are given as means ± s.d. for 5 individual experiments performed in duplicate. The differences between the mean values were evaluated by Student's *t* test.

RESULTS AND DISCUSSION

Human erythrocytes contain a number of proteases, both in the cytosolic and membrane fractions.[14–17] To exclude possible effects of cytosolic proteases,[14] isolated red cell membranes instead of erythrocytes[3,8] were used in this work. To provide direct evidence for the ability of insulin to activate a human red cell membrane protease, the effect of the hormone on the hydrolysis of BAPNA, a chromogenic protease substrate, by red cell membranes was tested. As shown in Table I (A) insu-

lin induced a significant (*ca*. 40 %) increase in the hydrolysis of BAPNA compared to the value measured in controls incubated without insulin. To gain insight into the specificity of the activated protease, the effect of TLCK, an active-site inhibitor of trypsin, on the protease activity was examined.[6] Table I (A) shows that the insulin-activated protease was selectively inhibited (up to 30 %) by TLCK, whereas the addition of TLCK did not affect the activity of the membrane protease of red cells incubated without insulin. Insulin-activation of red cell protease was a rapid process: the maximal activities were observed already after an incubation time of 20 min and did not decline upon prolonged incubation, up to 5 h.

TABLE I. Insulin-induced red cell membranes proteolytic activity (A) and GPI-Hb formation (B)

A. Proteolytic acitivty:		
Additions	Release of nitroaniline (nmol/min/mg MP)	
	No insulin	+ Insulin
None	2.5 ± 0.2	3.6 ± 0.2*
TLCK (30 μM	2.5 ± 0.2	3.2 ± 0.3
TLCK (60 μM)	2.4 ± 0.1	2.9 ± 0.2*
TLCK (120 μM)	2.4 ± 0.2	2.7 ± 0.2*
B. GPI-Hb formation:		
Additions	GPI-Hb (% of initially added HbAo)	
	No insulin	+ Insulin
None	n.d.	2.9 ± 0.2
TLCK (120 μM)	n.d.	1.6 ± 0.2*
BAME (50 μM)	n.d.	1.4 ± 0.1*

A. Membranes (5 mg MP/mL) were incubated with and without insulin (1 μg/mL) in the presence and absence of the indicated concentrations of TLCK in 60 mM sodium phosphate buffer pH 7.4 containing 10 μM ATP for 20 min at 37 ºC. The membranes were sedimented at 22000 g for 15 min at 4 ºC, washed three times in the same buffer (in which TLCK had been added to the concentration corresponding to that in the incubation mixture) and then, resuspended in 1 mL of the same buffer. Protease activity was estimated using the chromogenic substrate BAPNA as described in the Experimental.
B. Mixtures of membranes (5 mg MP/mL) and HbAo (30 mg/mL) were incubated with and without insulin (1 μg/mL) in the presence and absence of the indicated concentrations of TLCK and BAME in 60 mM sodium phosphate buffer pH 7.4 containing 10 μM ATP for 3 h at 37 ºC. The membranes were sedimented at 22000 g for 15 min at 4 ºC and the GPI-Hb was estimated in the supernatant as described in the Experimental procedures.
The results are the means ± s.d. of 5 individual experiments performed in duplicate; n.d. – non detectable. Values significantly different from the corresponding control values are indicated *p<0.01.

Cytosolic proteases were found to coelute with minor Hb fractions from the Bio-Rex column whereas the main HbAo remains devoid of proteolytic activity.[13]

To exclude the possible effect of cytosolic proteases on GPI-Hb formation, a purified HbAo fraction was used in the experiments designed for estimating the GPI-Hb formation. As shown in Table I (B), GPI-Hb (*ca.* 3 %) was formed upon incubation of red cell membranes and HbAo in the presence of insulin (but not in its absence). Inhibition of protease activity with TLCK parallels the decreased yield (by *ca.* 45 %) of GPI-Hb (Table I (B)). Addition of BAME, a protease substrate analog,[17] resulted also in a decreased yield (by *ca.* 50 %) of GPI-Hb. In contrast to insulin activation of protease, which was a fast process, the formation of GPI-Hb was much slower: the maximal yield of GPI-Hb was achieved after incubation for 5 h.

In summary, the presented results indicate that insulin activates a novel protease with trypsin-like specificity in human red cell membranes, which is related to insulin-activated protease in other cells.[6,17] In confirmation of the hypothesis of other authors,[7] we assume that the insulin receptor tyrosine kinase in red cell membranes stimulates the phosphorylation of a specific membrane associated protease, which activates the enzyme. Hemoglobin is known to interact with lipids from inner membrane surface of red cells.[18,19] The GPI transfer to the C termini of Hb β-chains may then be explained by assuming that the phosphate groups of the GPI-lipids which come in close contact with Hb serve as an affinity label, interacting with the positively charged groups at the 2,3-BPG binding site in the Hb molecule permitting the ethanolamine from GPI to come in close contact with the C-terminal part of the β polypeptide chain of the Hb molecule. In the lipid environment of the red-cell membrane, the activated protease is able to catalyze, albeit slowly, the transpeptidation, *i.e.*, the replacement of the carboxy-terminal amino acid(s) residues with GPI as an exogenous nucleophile. The candidate for the cleavage site is the peptide bond between the residues Lys-144 and Trp-145 in the β-chain of Hb, followed by GPI binding to the carboxy terminal of the Lys-144 residue and concomitant release of the C-terminal Trp-His dipeptide. Detailed studies of the insulin-activated red cell protease and the effects it produces on red cell proteins are currently under progress in our laboratory.

The mechanism of GPI addition to Hb described in this work may be relevant to other proteins as well. Thus, GPI addition to Hb was found to parallel its addition to some of the erythrocyte membrane proteins,[20] and insulin-induced hydrolysis of GPI-anchored proteins from the cell surface was found to precede their recovery upon prolonged insulin exposure.[21] Activation of protease was found to be associated with the activation of erythrocyte membrane phospholipase C (V. Niketić, unpublished observation). Human erythrocytes, which represent an extremely useful and easily accessible cellular model for the study of a variety of proteins, seem to be well suited also for studying the effects and the processes involved in cross-talk between GPIs and insulin.[22,23] The results described in the present paper may bear relevance to studies of physiological disorders that are characterized by hyperinsulinism, such as cases of diabetes and hypoglycemia.

ИЗВОД

КОВАЛЕНТНО ВЕЗИВАЊЕ ГЛИКОИНОЗИТОЛФОСФОЛИПИДА (GPI) ЗА ХЕМОГЛОБИН ПОД ДЕЈСТВОМ ИНСУЛИНА ПРАЋЕНО ЈЕ АКТИВИРАЊЕМ ПРОТЕАЗЕ ИЗ МЕМБРАНЕ ЕРИТРОЦИТА

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У нашим ранијим радовима показано је да у условима хиперинсулинизма и хипогликемије, *in vivo* и *in vitro*, долази до ковалентног везивања гликоинозитолфосфолипида (GPI) за карбоксилне крајеве оба β-низа молекула хемоглобина (Hb), што се манифестује настајањем нове, до тада непознате, мање фракције хемоглобина (GPI-Hb) (Niketić *et al.*, *Biochem. Biophys. Res. Commun.* **239** (1997) 435). У овом раду је показано да везивање инсулина за мембране еритроцита изазива активирање мембранске протеазе, те да је настајање GPI-Hb у корелацији са протеазном активношћу. Претпостављено је да протеаза активирана инсулином може, мада споро, да катализује реакцију транспептидације, тј. замену аминокиселинских остатака са карбоксилног краја β-низова молекула Hb са GPI-липидом као егзогеним нуклеофилом. Према нашем сазнању описани резултати први пута показују да инсулин стимулише протеазну активност у еритроцитима, те да је ова активност повезана са пост-транслационим везивањем GPI-липида за протеине.

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