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Insulin-induced lipid binding to hemoglobin

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Abstract: Under hypoglycemic conditions, concomitant hyperinsulinism causes an apparent modification of hemoglobin (Hb) which is manifested by its aggregation (Niketić *et al.*, *Clin. Chim. Acta* **197** (1991) 47). In the present work the causes and mechanisms underlying this Hb modification were studied. Hemoglobin isolated from normal erythrocytes incubated with insulin was analyzed by applying ^{31}P-spectrometry and lipid extraction and analysis. To study the dynamics of the plasma membrane during hyperinsulinism, a fluorescent lipid-analog was applied. In the presence of insulin, phosphatidylserine (PS), phosphatidylethanolamine (PE) and cholesterol were found to bind to Hb. Lipid binding resulted in Hb aggregation, a condition that can be reproduced when phospholipids are incubated with Hb *in vitro*. Using a fluorescent lipid-analog, it was also shown that exposing erythrocytes to supraphysiological concentrations of insulin *in vitro* resulted in the internalization of lipids. The results presented in this work may have relevance to cases of diabetes mellitus and hypoglycemia.

Keywords: insulin, erythrocyte, hypoglycemia, hyperinsulinism, membrane lipids, hemoglobin.

INTRODUCTION

Human red blood cells (RBC) are known to possess insulin receptors that bind insulin in a manner similar to that observed for other cell types.[1,2] The effects that insulin has upon erythrocytes at the molecular and/or structural level are largely unknown. We have demonstrated that under hypoglycemic conditions, the concomitant hyperinsulinism caused apparent modifications of hemoglobin (Hb). These modifications are represented by the aggregation of Hb and by the appearance of a minor Hb fraction, HbA1x (4 % of the total Hb).[3,4] The modification of Hb into HbA1x was shown to be due to covalent binding of glycoinositolphospholipid (GPI) derived from the RBC membrane to the C termini of both Hb β-chains.[5]

The present work was initiated in order to examine the underlying cause and mechanism of modification of Hb manifested by its aggregation. The molecular properties of he-

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moglobin from normal erythrocytes incubated with insulin were analyzed. The results demonstrate that supraphysiological concentrations of insulin induces lipid binding to Hb. Lipid binding causes the Hb to aggregate, a condition that can be simulated when phospholipids are incubated with Hb *in vitro*. Furthermore, using a fluorescent lipid-analog,[6,7] it was shown that lipid internalization occurs in erythrocytes in response to supraphysiological concentrations of insulin.

EXPERIMENTAL

Starting materials

Erythrocytes isolated[4] from freshly drawn blood samples of normal healthy volunteers were suspended (1:2) in buffer G, pH 7.4[1] from which bovine serum albumin was omitted since this protein was found to favor hemolysis upon prolonged incubation. Incubation with 1 μg/ml of porcine insulin (NOVO, Copenhagen, Denmark) was done at 37 ºC under agitation for 5 h. Controls without insulin were run in parallel. Insulin binding to the erythrocytes was estimated according to the method of Gambhir.[1] Hemolysis of the erythrocytes and separation of the Hb fractions by means of gel chromatography on a Sephadex G-100-120 column (Pharmacia) were as described previously.[4] For calibration of the column, glutaraldehyde-linked Hb oligomers of known molecular sizes[8] were used. The concentration of Hb was determined by CN-methemoglobin procedure as described by Tentori and Salveti.[9]

Incubation of Hb with phospholipids and cholesterol

The hemolysates prepared from normal erythrocytes were extensively dialyzed against distilled water followed by incubation with either phosphatidylserine (PS) or phosphatidylethanolamine (PE) alone or in combination with cholesterol (all from Sigma) in the following molar ratios: Hb:phospholipid 5:1; Hb:phospholipid:cholesterol 5:1:0.5. The lipids were dissolved in a 1:1 (v/v) mixture of Triton X-100 and 60 mM sodium phosphate (SP) buffer pH 7.4. Twenty μl of this solution were added to 1 ml of Hb solution (15 mg/ml) followed by incubation at 37 ºC for 3 h. The Hb solution (1 ml) containing 20 μl of Triton X-100 and SP buffer mixture was incubated as a control.

^{31}P-NMR Methods

For ^{31}P-NMR studies, Triton X-100 (to a final concentration of 5 %) was added to the Hb samples eluted from the Sephadex column. The ^{31}P-NMR spectra of membrane samples (containing 5 % Triton X-100) isolated from normal RBC[10] were used for comparison. The NMR spectral data were obtained on a Bruker MSL 400 instrument operating in the Fourier transform mode at 161.981 MHz. All experiments were carried out at 22 ºC under full broad-band decoupling conditions. The number of transitions that were accumulated was 40,000. All chemical shifts are reported relative to external 85 % phosphoric acid.

Lipid extraction and analysis

The Hb fractions eluted from the Sephadex column were lyophilized, dissolved in water (60–130 mg in 0.6 ml of water) and the lipids were extracted according to the procedure of Bligh and Dyer.[11] The lipid phosphorus levels were determined as described by Bottcher.[12] The cholesterol levels were estimated by an enzymatic assay using a commercial kit (Human GmbH). Thin layer chromatography (TLC) was performed on Silica Gel 60 HPTLC plates (Merck) using $CHCl_3$/ CH_3OH/ 20 % NH_4OH (70:30:5) as the solvent system.

Insertion of fluorescent lipids and lipid internalization

To insert the fluorescent lipid analogue 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine (C_6-NBD-PC) (Avanti Biochemicals) into the erythrocyte plasma membrane, a procedure similar to that described by Kok *et al.*[6] was applied. The lipid (15 nmol) (stored in chloroform/methanol (2:1)) was dried under nitrogen and subsequently solubilized in 50 μl of absolute ethanol. The ethanolic lipid solution was rapidly injected into a solution of 1 ml 120 mM KCl/ 30 mM NaCl/ 10 mM Na_2HPO_4

(KNP) buffer pH 7.4, under vigorous vortexing. This solution was then added to a tube containing 5×10^9 /ml erythrocytes in 9 ml KNP buffer. Labeling was carried out for 30 min at 37 ºC with mild shaking. Non-inserted (free) lipid was removed by washing the erythrocytes. The cells were resuspended in 25 ml of incubation buffer to a final erythrocyte concentration of 2×10^8 /ml. To initiate the internalization of the membrane-inserted lipid, the cells were incubated with insulin (1 μg/ml) as described above. A control without insulin was run in parallel.

Back-exchange of membrane inserted lipid

Back-exchange was carried out by incubating erythrocytes with small unilamellar vesicles of dioleylphosphatidylcholine (DOPC SUV). The liposomes were prepared by suspending the dried lipids in KNP buffer followed by sonication for 15 to 30 min. Twenty microlitre of 8 mM DOPC SUV (500 mnmol/ml) was added to 1 ml of erythrocyte suspension and incubated with continuous rotation for 15 min at room temperature. The erythrocytes and liposomes were separated by centrifugation. Subsequently, the cells were subjected to another round of back-exchange. The fluorescence in the pooled supernatants (back-exchange fraction) and cells was determined after addition of Triton X-100, 1 % v/v. The fluorescence was measured using a Perkin-Elmer MPF-43 fluorescence spectrophotometer. For C_6-NBD-PC, the excitation and emission wavelengths were 465 and 530 nm, respectively.

Microscopy

Fluorescence microscopic examination was performed with a Leitz Orthoplan microscope equipped with a Leitz Vario Orthomat 2 photographic system and the following filter set: blue excitation, BP 450 to 490/LP515 (NBD channel). The photomicrographs were taken at 30 s exposure times, using a Kodak T-max P3200 film that was processed at 12800 ASA.

RESULTS

Previous gel filtration studies demonstrated that under hypoglycemic conditions concomitant hyperinsulinism caused, both *in vivo* and *in vitro* apparent modifications which were manifested by the aggregation of Hb.[4] ^{31}P measurements demonstrated that the Hb aggregates from normal RBC incubated with insulin eluted from a Sephadex column contained phospholipids (PL). No phospholipids were detected in Hb from control samples.

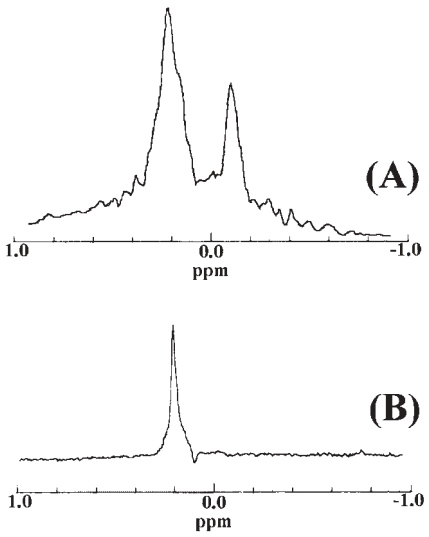

Fig. 1. ^{31}P-NMR spectra of soluble red cell membranes and Hb aggregates. (A) soluble red cell membranes to saturation in 5 % Triton X-100, pH 7.1. (B) Hb aggregates isolated by means of Sephadex column from normal RBC incubated with insulin (25 mg/ml) in 5 % Triton X-100, pH 7.0. For experimental details see the Experimental section.

Figure 1 compares the representative ^{31}P spectra of RBC membranes (pH 7.1) and Hb aggregates (pH 7.0) from normal erythrocytes incubated with insulin. The striking difference between the two spectra is the lack of the signal resonating at – 0.15 ppm in the spectrum of Hb aggregates. By comparison with published phospholipid chemical shift data,[13,14] the resonance at 0.2 ppm is assigned to PE and the resonance at – 0.15 ppm is assigned to PC. However, because of the similarity in ^{31}P chemical shift for these lipids, the ^{31}P resonance from PS is certainly represented in the 0.2 ppm resonance.

The tight binding of PL to Hb in Hb aggregates is demonstrated by the fact that it is impossible to remove the PL by gentle ultrafiltration techniques. However, extensive dialysis resulted in the partial removal of the bound PL. The binding of proteins to negatively charged lipids is predominantly electrostatic in nature, and is reduced by increasing the ionic strength.[15,16] Indeed, elution of the Sephadex column with buffer or salt solutions such as 0.15 M NaCl resulted in the dissociation of the Hb aggregates. Under these conditions, Hb from insulin-treated cells eluted as a single peak. This elution profile is similar to that of Hb from untreated cells (data not shown).

The Hb aggregates, recovered by gel filtration as described above, were further analyzed to determine the nature of the PL present. The lipids were extracted and a qualitative assessment of the lipid composition was achieved by thin layer chromatography (data not shown). Only PE and PS were detected in the Hb aggregates derived from erythrocytes that had been exposed to insulin. A quantitative determination of the phospholipid (phosphorus assay) and cholesterol levels from the various samples of Hb aggregates revealed the association of *ca.* 0.1 mol of phospholipid and 0.05 mol of cholesterol per mole of Hb in cells treated with insulin.

The association of lipids with Hb as revealed by the above data may suggest that lipids play an active role in causing the aggregation of Hb under conditions of hyperinsulinism. To obtain more direct experimental support for such a mechanism, Hb isolated from normal RBC was incubated with either PS or PE alone or in combination with cholesterol, followed by gel chromatography. Under the applied experimental conditions, PS and PE induced aggregates of a size comparable to that of trimers and a mixture of dimers and monomers, respectively (Fig. 2). Addition of cholesterol caused an increase in the elu-

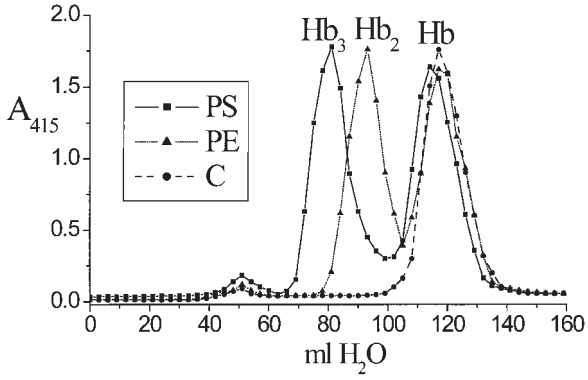

Fig. 2. Elution pattern of hemoglobin incubated with phosphatidylserine (PS) or phosphatidylethanolamine (PE) on Sephadex G-100-120 column. Hemolysates from normal erythrocytes were prepared and incubated with lipids as described in Experimental section. Experimental conditions: 0.5 ml of hemolysate containing about 40 mg of Hb was applied on a 1.5×75 cm column equilibrated and eluted with distilled water at a flow rate of 20 ml/h. Fractions of 3 ml were collected.

tion volumes for Hb fractions containing phospholipids (results not shown), indicative of smaller aggregates.

Internalization of the erythrocyte membrane

Hb could acquire lipids either by direct monomeric transfer of the lipid between the plasma membrane and the protein, or after internalization of the membrane by invagination. To gain further insight, a fluorescent lipid analogue, C_6-NBD-PC, was employed to study the dynamics of the plasma membrane during hyperinsulinism. The analogue was inserted into the outer leaflet of the erythrocyte membrane as described in the Methods. Its exclusive presence in the outer leaflet was confirmed by demonstrating that the entire membrane-inserted lipid fraction could be removed by back-exchange with unlabeled lipid vesicles (*cf.* Fig. 4c (control)). When C_6-NBD-PC labeled cells were subsequently incubated with insulin in G-buffer, internalization of the analogue was observed. Back-exchange revealed that approximately 10 % of the plasma membrane fraction had internalized during a 5 h incubation period at 37 ºC (Fig. 3).

Fluorescence microscopy revealed that the internalized fraction of the lipid analogue was present in distinct spots, presumably representing intracellular vesicular structures. Usually, three to four of such structures per erythrocyte were detected (Fig. 4). Their ap-

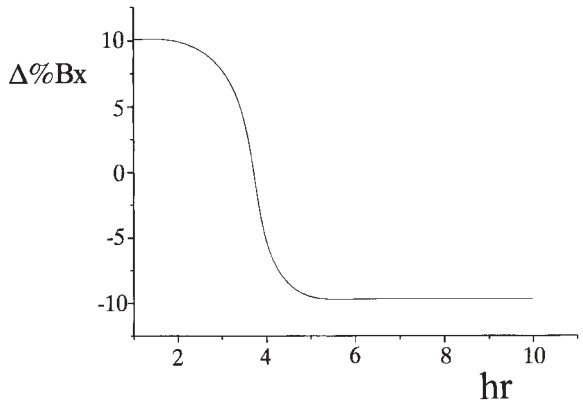

Fig. 3. Kinetics of internalization of the fluorescent lipid analog as revealed from the difference in the fluorescence of the back-exchange fraction (Δ %Bx) from C_6-NBC-PC labeled erythrocytes incubated with insulin and the respective control. For experimental details see the Experimental section.

pearance was a gradual process, the first vesicles appearing after three hours of incubation. After 5 h of incubation, the process appears completed as, reproducibly, no further increase in the number of vesicles (beyond 3–4) was seen. Note that the kinetics of vesicle appearance is in accordance with the kinetics of lipid internalization, as presented in Fig. 3.

DISCUSSION

In this work evidence is presented that supraphysiological concentrations of insulin can cause interactions between lipids derived from RBC membranes and Hb. These interactions occurred under conditions of low ionic strength and resulted in the formation of loosely associated Hb aggregates.

Under conditions of hyperinsulinism, down regulation of the receptor occurs, causing its internalization.[17] Under such conditions it was found that PE, PS and cholesterol (at a

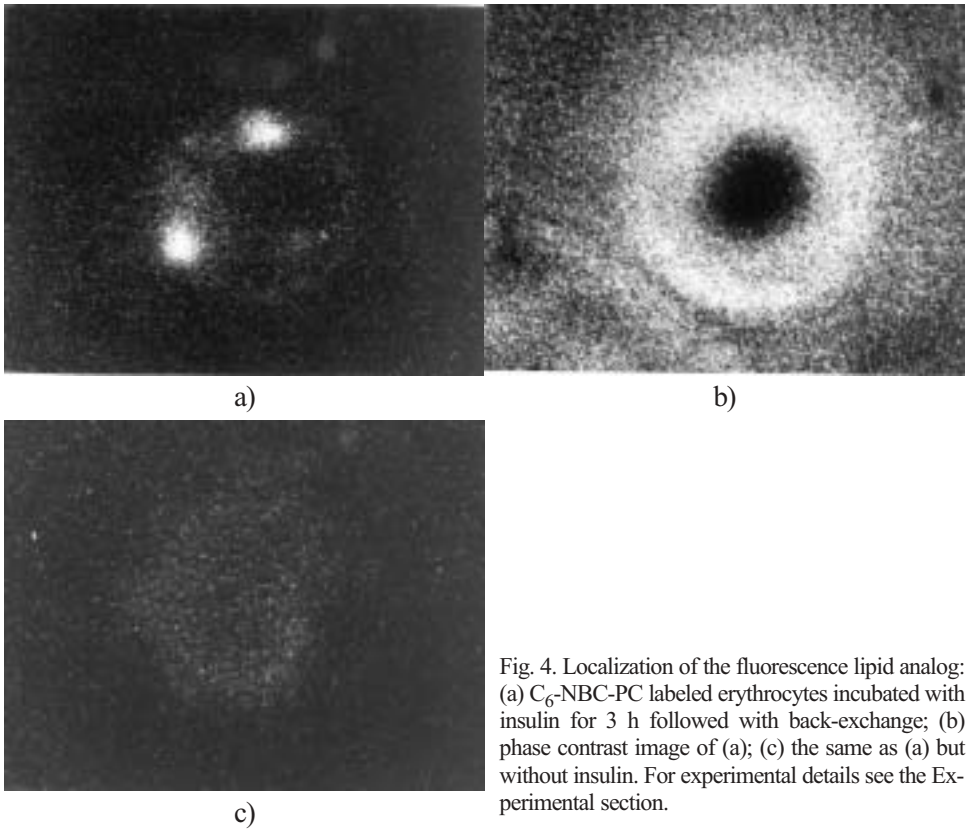

Fig. 4. Localization of the fluorescence lipid analog: (a) C_6-NBC-PC labeled erythrocytes incubated with insulin for 3 h followed with back-exchange; (b) phase contrast image of (a); (c) the same as (a) but without insulin. For experimental details see the Experimental section.

phospholipid to cholesterol ratio of *ca.* 1:0.5) were bound to Hb, and, most likely, cause the protein to aggregate. The latter is supported by experiments which demonstrated that exogenous addition of these lipids can simulate such an event. Application of the fluorescent lipid analogue (C_6-NBD-PC) allowed the ready detection and visualization of the internalization of labelled lipids integrated into the membrane in the cell. Approximately 10 % of the total C_6-NBD-PC pool became internalized over a period of approx. 5 h This percentage is consistent with the fraction of internalized membrane-lipids associated with Hb (in aggregates). That is, given the amount of Hb in RBC (3 μmol/ml packed RBC) and the amounts of lipid in the RBC membrane (1.6–2.3 μmol/ml RBC of phospholipids and 1.7–2.1 μmol/ml RBC of cholesterol),[18] it was calculated that approximately 15 % of the membrane lipids were internalized in RBC exposed to insulin. Therefore, this energy dependent internalization of insulin receptors in erythrocytes (shown previously to occur in response to exposure of erythrocytes to high concentrations of insulin)[17] is accompanied by a concomitant internalization of the membrane lipids.

Given the monomeric exchange properties of the fluorescent lipid analogue and in light of its restricted localization to defined areas within the cell, a vesicular internalization mechanism is suggested. The C_6-NBD-PC lipid appeared to be restricted to the inner leaf-

let of the internalized vesicle. In this regard, these findings support previous suggestions that internalized insulin receptors are present in inside-out vesicles.[19] Accordingly, at the leaflet facing the cytoplasm, PS and PE will be exposed, as these lipids are predominantly restricted to the inner leaflet of the erythrocyte membrane. Since C_6-NBD-PC is not found associated with Hb in Hb aggregates, PS, PE and cholesterol likely gain access to Hb as monomers, rather than as whole vesicles acting as nucleation sites for Hb. It was found that apohemoglobin causes the disruption of PS vesicles and the formation of micellar protein/lipid complexes.[20] It seems reasonable to suggest that upon prolonged exposure, PS (and also PE), which can no longer engage in dynamic interactions with the cytoskeleton, may become prone to 'exchange' with Hb, which itself contains a high-affinity binding site for PS (see below). Consequently this scenario leads to Hb aggregation and the likely destruction of the internalized vesicles.

Using RBC membranes and lyposomes, numerous studies have demonstrated that Hb binds to negatively charged phospholipids. These interactions are reduced as the pH is increased, but still exist in the physiological pH range.[21] Hemoglobin/phospholipid interactions are characterized initially by very rapid electrostatic, followed by hydrophobic interactions.[21–23] The nature of the negatively charged phospholipids is important in promoting the interaction with Hb. For example, the effect of phosphatidic acid is greater than that of phosphatidylinositol which is congruent to that of phosphatidylglycerol being greater than that of phosphatidylserine.[24] Cholesterol inhibits both the hydrophobic and ionic interactions between Hb and the phospholipid[21,25] which may explain the findings that the presence of cholesterol resulted in a decrease in the size of the Hb aggregates.

Interaction of hemoglobin with negatively charged phospholipids induces Hb oxidation, the dissociation of the heme-globin complex and the denaturation of Hb.[24–26] For Hb aggregates isolated from insulin-treated RBC, a blue shift in the Soret band from 416 to 414 nm along with the appearance of a small peak at 650 nm characteristic of iron-free porphyrin were observed. Taken together, these data suggest the dissociation of the heme-globin complex and subsequent removal of iron from the porphyrin ring.[24] Phospholipid-induced Hb oxidation can be associated with lipid peroxidation.[27,28] Indeed, the formation of thiobarbituric acid-reactive substances (TBARS)[28] was found to be approximately 1.5 times greater in insulin-treated RBC than in the respective control.

The results described in the present work may bear relevance to studies of physiological disorders that are characterized by hyperinsulinism. Such diseases include cases of diabetes mellitus and hypoglycemia.[29–31] Insulin-induced lipid binding to Hb may represent one of the sources of free radicals in insulin-treated diabetic patients who have been generally described as being under enhanced oxidative stress.[30] Aggregation of Hb can be detected 10 days after pancreotomy in hypoglycemic patients after the normal concentrations of insulin and glucose have been reestablished.[4] Hyperinsulinism triggered, apart from Hb aggregation, the formation of a GPI adduct of Hb.[8] The GPI adduct of Hb could be detected even 30 days after pancreotomy.[4] The detection and monitoring of chronic hyperinsulinism could be improved and/or facilitated by a means to identify the existence

(and consequently levels) of not only Hb aggregates but also the GPI adduct of Hb. Future work, employing the current system and approach will contribute to unraveling the underlying mechanism by which Hb acquires lipids under hyperinsulinism and hypoglycemia conditions.

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ИЗВОД

ВЕЗИВАЊЕ ЛИПИДА ЗА ХЕМОГЛОБИН ПОД ДЕЈСТВОМ ИНСУЛИНА

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У ранијим радовима је показано да хиперинсулинизам у условима хипогликемије изазива модификацију молекула хемоглобина која се манифестује његовим агрегирањем (Niketić *et al., Clin. Chim. Acta* **197** (1991) 47). У овом раду испитивана је ова модификација молекула хемоглобина, као и механизам њеног настајања. Применом ^{31}P-спектрометрије и анализом липидног екстракта утврђено је да у нормалним еритроцитима инкубираним са инсулином долази до везивања фосфатидил-серина, фосфатидил-етанолмина и холестерола за молекул хемоглобина. Везивање фосфолипида за хемоглобин доводи до његовог агрегирања што је потврђено експериментима у којима је хемоглобин инкубиран са фосфолипидима *in vitro*. Применом флуоресцентног липидног аналога показано је да при излагању еритроцита супрафизиолошким концентрацијама инсулина долази до интернализације мембранских липида. Добијени резултати могу бити од значаја за пацијенте оболеле од шећерне болести и хипогликемије.

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