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Cholesterol bound to hemoglobin in normal human erythrocytes: a new form of cholesterol in circulation?

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Abstract

Objective: To study lipid fraction that is occasionally observed in red blood cell (RBC) hemolysate (supernatants from which membranes were separated).

Study design: Plasma lipid profiles, cholesterol (Ch) and phospholipids (PL) in intact RBCs, RBC membranes and hemolysates were examined in young healthy male population in winter and summer.

Results: The RBC Ch and PL content was significantly higher than in membranes, both in winter and summer. The ''excess'' of cholesterol (associated with phospholipid) was bound to hemoglobin yielding Hb–lipid adduct (Hb–Ch), the pools in the RBC membrane remaining virtually unaltered. Levels of hemoglobin–lipid complex (Hb–Ch), which were significantly higher in winter than in summer (30% and 19% of the total Hb, respectively), positively correlated with plasma HDL cholesterol levels.

Conclusion: To our knowledge, this is the first demonstration of cholesterol binding to Hb. The results suggest influence of plasma lipoprotein metabolism on the formation of Hb–Ch.

Keywords: Cholesterol; HDL cholesterol; Hemoglobin; Red blood cells; Hemoglobin–lipid; Seasonal variation

Introduction

Red blood cell (RBC) cholesterol (Ch) and phospholipid (PL) content have been frequently estimated under normal as well as under various pathological conditions. It is generally accepted that lipids extracted from intact RBCs originate from the red cell membrane, so that intact RBC lipid extracts are often applied for the membrane lipid assays. Reviewing the published data, we noticed that reported values for the total cholesterol and phospholipid content in normal human RBC vary greatly (see Refs. [1–6]), which is usually ascribed to the differences in extraction procedures and red blood cell counting [1,7]. However, by specifically comparing values from studies that applied similar extraction procedures, we still found substantial variation in reported values. Thus, by analyzing groups consisting of hematologically normal subjects of both genders and various ages, Reed et al. [2] reported values of 3.6 mmol Ch/l and 4.4 PL/l RBC with a range of 3.4–3.8 mmol Ch/l RBC and 4.1–4.8 mmol PL/l RBC, whereas considerably higher and more variable RBC lipid content were found in other studies that applied similar extraction procedures: approximately 4.5 mmol Ch/l RBC and 5.5 mmol PL/l RBC [3–5] (to compare data from cited studies with those from Ref. [2], we expressed the originally reported value per liter of RBC—12×10^{12} RBC/l).

During our previous investigations concerning insulin-induced lipid binding to hemoglobin (Hb) [8,9,10], the need arose to estimate lipids in erythrocytes from young healthy males. In contrast to the RBC samples that we used in previous experiments, we occasionally observed in hemolysates (supernatants from hemolysed RBCs from which membranes were separated) of these RBCs the presence of an ''excess'' of cholesterol that did not sediment on centrifuging under conditions used for red cell membrane isolation [1]. Incomplete sedimentation of RBC lipids was reported in early studies; for example, in hemolysed pig erythrocytes 41% of

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the lipid phosphorus was found not to sediment upon centrifuging at 36,000–90,000 × *g* [11].

This prompted us to initiate the present pilot study with the aim of providing further data concerning the normal RBC lipids to better clarify the phospholipid and cholesterol content in normal RBCs. We addressed this question by analyzing the RBC cholesterol and phospholipid content in a group of young healthy males of the same geographical area (Belgrade) with similar dietary habits in two seasons: January (winter) and July (summer).

Our results demonstrate that, in contrast to the cholesterol and phospholipid contents of red cell membrane that are confined to a narrow interval, the lipid levels estimated in intact RBCs showed substantial variation: the lowest individual values for RBC lipids corresponded to those found in membrane, whereas in RBCs with higher lipid contents, the "excess" was found in hemolysates. By applying simple biochemical procedures, it was documented that this "excess" of cholesterol (associated with phospholipid) is bound to hemoglobin yielding Hb–lipid adduct (Hb–Ch) with binding stoichiometry of about two molecules of each Ch and PL per one Hb molecule in Hb–Ch. Significantly higher Hb–Ch levels in winter comparing to those in summer, which parallels plasma cholesterol levels as well as positive correlation between percent Hb–Ch and HDL cholesterol levels, were demonstrated, which suggest the influence of plasma lipoprotein metabolism on the formation of Hb–Ch.

Methods

Thirty-six young males (ages 19–27) were included in this study. They were students of our institution and individuals on a regular physical examination before military service who gave informed consent to participate in our study. They were apparently healthy, maintaining their usual diets, and were free of clinical evidence of any chronic illness. The exclusion criterion was elevated plasma lipids (cholesterol >5.2 mmol/l and/or triglycerides >1.5 mmol/l). The study took place in the months of January (winter) and July (summer). A detailed interview of all participants was performed to evaluate their dietary habits by indirect calculation of the diet composition of the previous 2 weeks.

Venous blood was collected after 12 h of overnight fasting and mixed with an anticoagulant (4.5 ml of blood added to 0.5 ml of 3.8% sodium citrate solution). RBCs were treated within 1 h after blood sampling and analytical procedures were completed the next day.

Cholesterol and triglyceride (TG) concentrations were determined using enzymatic assay kits obtained from Reanal (Hungary) and Randox (UK), respectively (CV for triglycerides: 3.1%; CV for cholesterol: 2.6%). HDL cholesterol was measured in the supernatant following the precipitation of ApoB-containing lipoproteins with dextran sulfate and $MgCl_2$ [12]. LDL cholesterol was calculated using the Friedewald equation [13]. Plasma was extracted with ethanol/ether (3:1; v/v) for measurement of lipid phosphorus [14]. Erythrocytes were separated by centrifugation at 4 °C, the plasma and buffy coat were removed, and the cells were washed three times with cold isotonic saline. A portion of the cells was hemolysed with 30 volumes of cold 10 mM Tris–HCl buffer, pH 7.4 [1], and an aliquot of cell hemolysate was taken for the total RBC lipid extraction [2]. Membranes were separated from the rest by centrifugation at 20,000 × *g* for 40 min [1]. The hemolysate and the membranes (suspended in an equal volume of 10 mM Tris–HCl buffer, pH 7.4), were kept at 4 °C for further analysis. An aliquot of membrane suspension was used for membrane protein (MP) concentration determination by a modified method of Lowry [15] using bovine serum albumin as a standard. Lipids were extracted from the second portion following procedure described in Ref. [2]. One aliquot of hemolysate was used for estimation of hemoglobin [16]. A second aliquot was taken for chromatography on Sephadex column (see bellow) while the lipids were extracted [2] from the third portion. Aliquots of lipid extracts were taken for cholesterol and phospholipid determinations, which were carried out in triplicate following the procedures outlined above. Hemolysates were subjected to chromatography on a Sephadex G-100-120 column (Pharmacia, Sweden) as described previously [8,10] and separated Hb fractions were concentrated by ultrafiltration in an Amicon model-8050 50-ml ultrafiltration cell with gentle stirring. Lipids from concentrated Hb fractions eluted from Sephadex column were extracted and analyzed using the procedures outlined above.

Incubation of Hb with cholesterol and phospholpids was performed as described previously [10]. For these experiments, Hb samples were first subjected to extensive dialysis against 60 mM sodium phosphate buffer (pH 7.4), followed by dialysis against distilled water. The hemolysates were than incubated either with cholesterol alone or combined with phosphatidylserine (all from Sigma) in the following molar ratio: Hb/cholesterol 1:5; Hb/cholesterol/phospholipid 1:1:1. The lipids were dissolved in a 1:1 (v/v) mixture of Triton X-100 and 60 mM sodium phosphate buffer pH 7.4. Twenty microliters of this solution was added to 1 ml of Hb solution (ca. 90 mg/ml) followed by incubation at room temperature for 24 h and repeated extraction of unbound lipids with equal volumes of carbontetrachloride.

All results are expressed as means ± SD. Values of lipid determinations represent the average of three separate analyses. Statistical evaluation of the data was performed by Student's *t* test and by linear regression analysis.

Results

Plasma lipid concentrations

Plasma lipid concentrations of the 36 subjects are shown in Table 1. Significant differences were found in total

Table 1
Seasonal variations in plasma lipid concentrations (n = 36)

Measurement	Summer (July)	Winter (January)
	mmol/l	
Total cholesterol	3.92 ± 0.31	4.21 ± 0.35*
LDL cholesterol	2.43 ± 0.28	2.58 ± 0.34*
HDL cholesterol	1.01 ± 0.16	1.16 ± 0.14*
Triglycerides	1.04 ± 0.35	1.05 ± 0.34
Phospholipids	2.19 ± 0.16	2.52 ± 0.15*

Results are expressed as means ± SD.
* $P < 0.001$.

cholesterol (TC) and LDL and HDL cholesterol, all of these being increased in the winter. The results are consistent with seasonal variations in plasma cholesterol levels that have been repeatedly reported, with the highest levels in winter and the lowest in summer [17–19].

RBC lipid concentrations

In the present study, RBC lipids were extracted by applying a slightly modified procedure of Reed et al. [2], which requires several extraction steps. Compared to other procedures tested [7,20], it yielded the best completeness of extraction both from intact RBCs and isolated membranes. Table 2 shows the values estimated for RBC cholesterol and phospholipids.

In contrast to the cholesterol and phospholipid contents of red cell membrane, which are confined to a narrow interval, the lipid levels estimated in intact RBCs were subjected to more variation: the lowest individual values for RBC lipids corresponded to those found in membrane, whereas in samples with higher lipid contents the "excess" was found in hemolysates (Table 2). The lipid content estimated in hemolysate represented approximately 50% of the total RBC lipid content in winter and 40% in summer. The characteristic molar ratio (Ch/PL = 0.83) of intact RBC was identical to that estimated for RBC membrane and hemolysates. RBC lipid content was about 20% higher in winter, which is exclusively due to the increased lipid content in RBC hemolysates. Cholesterol and phospholipids in hemolysates were associated with hemoglobin, yielding hemoglobin–lipid adduct (Hb–Ch; see below). Significantly higher lipid levels in RBC hemolysates in winter comparing to those in summer corresponded entirely to the increase in Hb–Ch content (Table 2).

Excess of red cell cholesterol and phospholipids in RBC is bound to hemoglobin

We previously observed that phospholipid binding to Hb results in the formation of loosely bound Hb aggregates, which elute from Sephadex column before the peak of normal Hb if the column is equilibrated and eluted with distilled water [8,10]. Fig. 1 shows chromatograms of normal hemolysates from this study, which contained cholesterol in addition to phospholipids, and reveals the presence of hemoglobin fraction (Hb–Ch, 0.2–55.0% of the total Hb), which elutes after the normal hemoglobin fraction (Hb). Hemoglobin samples preequilibrated with a mixture of cholesterol and phospholipid or with cholesterol alone exhibited similar behavior (Fig. 1). Adsorption of Hb–Ch to the Sephadex column was avoided if buffer or salt solution, such as 0.155 M NaCl, were used for column equilibration and elution: under these conditions, hemoglobin was eluted in the single peak with an elution volume characteristic for normal Hb tetramer.

Hemoglobin fractions, recovered from Sephadex column, were concentrated by ultrafiltration and further analyzed by carrying out lipid extractions of the samples. Small amounts of lipids were found associated with Hb fraction (0.04–0.10 mol Ch/mol Hb and 0.06–0.15 mol PL/mol Hb). Nearly all the phospholipid and cholesterol present in hemolysates was found to be associated with Hb–Ch fraction. Stoichiometry of lipid binding to Hb in Hb–Ch was approximately 1.9 ± 0.1 mol Ch/mol Hb–Ch and 2.1 ± 0.1 mol PL/mol Hb–Ch (n = 7).

Cholesterol bound to hemoglobin could not be removed either by extensive dialysis against distilled water and low ionic strength buffer such as 10 mM Tris–HCl buffer, pH 7.4, or by extraction with CCl_4. Under these conditions, about 80% of phospholipids dissociated from Hb–Ch. This suggests a higher affinity of cholesterol binding to Hb in Hb–Ch compared to that of phospholipids. Cholesterol bound to Hb in Hb–Ch could be removed substantially (up to 80%) by extensive dialysis against a buffer of higher

Table 2
RBC cholesterol and phospholipid contents (n = 36)

	Summer (range)	Winter (range)
Intact RBC (mmol/l RBC)		
Cholesterol	4.04 ± 0.41	4.99 ± 0.58*
	(2.54–4.96)	(2.55–7.08)
Phospholipids	4.88 ± 0.48	6.04 ± 0.67*
	(3.10–5.95)	(3.17–8.35)
Molar ratio Ch/PL	0.83 ± 0.05	0.83 ± 0.06
RBC membranes		
Cholesterol (mmol/l RBC)	2.44 ± 0.12	2.47 ± 0.14
(μmol/mg MP)	0.51 ± 0.03	0.51 ± 0.03
Phospholipids (mmol/l RBC)	2.97 ± 0.14	3.00 ± 0.17
(μmol/mg MP)	0.62 ± 0.03	0.63 ± 0.04
Molar ratio Ch/PL	0.82 ± 0.03	0.82 ± 0.04
RBC hemolysate (mmol/l RBC)		
Cholesterol	1.66 ± 0.46	2.62 ± 0.54*
	(0.17–2.47)	(0.18–4.70)
Phospholipid	2.01 ± 0.55	3.15 ± 0.61*
	(0.25–3.01)	(0.25–5.80)
Molar ratio Ch/PL	0.82 ± 0.06	0.83 ± 0.07
Hb–Ch (% of total Hb)	19.3 ± 5.4	30.5 ± 6.3*
	(0.2–28.7)	(0.2–55.0)

Results are expressed as means ± SD; RBC lipid levels are expressed per liter of RBC (12×10^{12} RBC/l).
* $P < 0.001$.

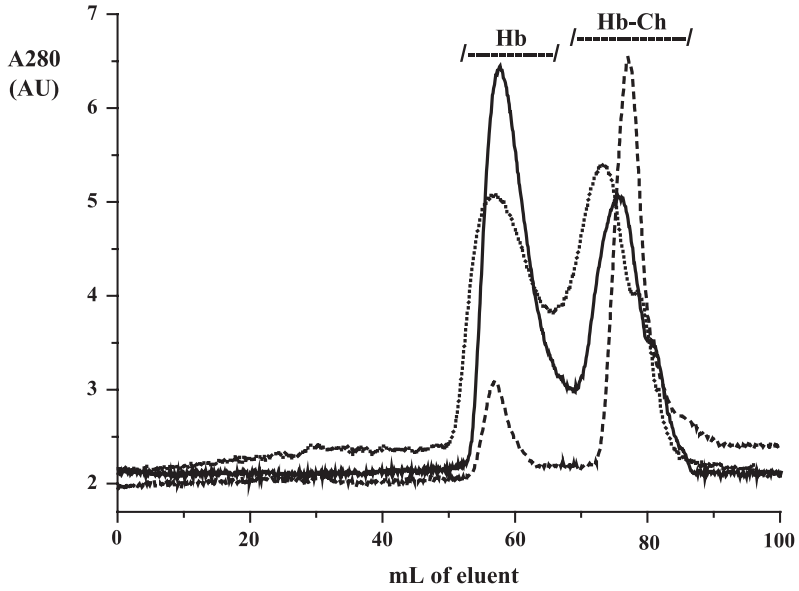

Fig. 1. Separation of hemoglobin samples on Sephadex G-100-120. The column (1.4 × 68 cm) was equilibrated and eluted with distilled water at flow rate 15 ml/h, and fractions of 2.5 ml were collected: red cell hemolysate (ca. 20 mg Hb) (—); hemoglobin (ca. 20 mg), which was previously equilibrated with either cholesterol/phosphatidylserine (PS) mixture in the molar ratio Hb/Ch/PS = 1:1:1 (· · ·) or with cholesterol alone in the molar ratio Hb/Ch = 1:5 (- - - -). For experimental details, see Methods.

ionic strength, such as 60 mM sodium phosphate buffer (pH 7.4). Hemoglobin samples preequilibrated with cholesterol–phospholipid mixture or with cholesterol alone exhibited similar behavior.

Correlation between Hb–Ch and plasma cholesterol levels

To investigate the possible relationship between "excess" of RBC cholesterol bound to Hb in Hb–Ch and plasma cholesterol levels, we assessed the correlation of Hb–Ch with plasma levels of total, LDL and HDL cholesterol. The Hb–Ch levels were significantly correlated only with plasma HDL cholesterol levels, both in summer (r = 0.54; $P < 0.01$) and in winter (r = 0.57; $P < 0.01$).

Discussion

The present study demonstrates that, in contrast to the cholesterol and phospholipid content of red cell membrane, which are confined to a narrow interval, the lipid content estimated in intact RBCs of investigated young healthy males was more variable. The lowest individual values for RBC lipids corresponded to those found in RBC membrane, whereas in samples with higher lipid contents, the "excess" was found in hemolysate bound to hemoglobin yielding Hb–lipid adduct (0.2–55.0% of total Hb).

The values for the total RBC lipid content of young males estimated in the present study are substantially higher and more variable than those reported in the original work of Reed et al. [2], but are close to those reported in other studies, which applied similar extraction procedure [3–5]. The source of the observed variabilities in RBC lipid content cannot be explained fully now. The effect of age [6] and diet [21] on total RBC lipid content has been demonstrated previously, and seasonal variations in RBC lipid content have been documented in this work.

The major finding of the present study is the detection of specific cholesterol (associated with phospholipid) binding to hemoglobin yielding Hb–lipid adduct. Our results indicate that Hb–Ch is formed in this group of healthy males under the conditions of elevated plasma cholesterol levels that occur because of season-related changes of plasma lipoprotein metabolism [17,18]. Total dietary fat and, to a greater extent, saturated fat and cholesterol were found to be related to seasonal changes in serum cholesterol [22]. Indeed, dietary intake of meat, eggs and diary products of our subjects were notably higher in winter than in summer. Our (preliminary) calculations show that fat provided ca. 36% (ca. 18% saturated fatty acids) of total (11,500 kJ) daily energy intake in winter and ca. 30% (ca. 14% saturated fatty acids) of total (11,300 kJ) in summer. Daily intake of cholesterol decreased from ca. 650 mg in winter to ca. 480 mg in summer.

Significantly higher Hb–Ch levels in winter comparing to those in summer that parallels plasma cholesterol levels as well as positive correlation between percent Hb–Ch and HDL cholesterol levels suggests an influence of plasma lipoprotein metabolism on the formation of Hb–Ch. However, the molecular mechanism resulting in a net transfer of lipids to Hb remains to be worked out, and that will probably be difficult.

It is premature to speculate on the physiological significance of Hb–Ch adduct described in this work. A positive relationship between the total cholesterol and LDL cholesterol levels and a negative relationship between HDL cholesterol levels and the risk of developing coronary artery disease were well established [23,24]. The reduction of total serum cholesterol concentrations has also become a major public health objective [25]. It is tempting to speculate that Hb–Ch represents a new form of cholesterol in circulation, which contributes to the permanent removal of the excess of unesterified cholesterol from circulation. This way, the RBCs may represent a part of the mechanisms involved in the first line "defense" against an excess of free cholesterol in circulation. Whether detection and estimation of Hb–Ch may represent useful additional parameter to routinely applied plasma total, LDL and HDL cholesterol measurements remains to be established. Indeed, we found significantly higher levels of Hb–Ch levels in small group of individuals with risk for cardiovascular disease (LDL Ch/HDL Ch > 3) [25] compared to Hb–Ch values reported in this work for healthy individuals. Structural and functional studies of hemoglobin–cholesterol interactions are pursued currently in our laboratory using both experimental and computational modeling techniques.

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