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INTERACTION OF PLASMA LIPOPROTEINS WITH ERYTHROCYTES

II. MODULATION OF MEMBRANE-ASSOCIATED ENZYMES

DAVID Y. HUI and JUDITH A.K. HARMONY *

Chemistry Department, Indiana University, Bloomington, IN 47401 (U.S.A.)

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Summary

When incubated with intact erythrocytes, low density lipoproteins (LDL) decrease the phosphate content of erythrocyte spectrin allowing the cells to undergo morphological transformation. The phosphate content of spectrin depends on the balance between the activity of membrane-associated cyclic AMP-independent protein kinases and phosphoprotein phosphates. LDL do not influence the activity of membrane-associated cyclic AMP-independent protein kinases; these lipoproteins activate by 2-fold and greater membrane-associated phosphatases as determined by hydrolysis of *p*-nitrophenyl phosphate and by phosphate hydrolysis of phosphorylated erythrocyte membrane proteins. We conclude that LDL interact at the exterior surface of the erythrocyte to stimulate dephosphorylation of spectrin. The significance of this conclusion is augmented by the fact that spectrin, the target for LDL-induced dephosphorylation, specifies cell morphology and modulates the distribution of cell-surface receptors. LDL also render erythrocyte acetylcholinesterase less susceptible to inhibition by F^-. Lipoproteins in the high density class (HDL) do not stimulate dephosphorylation of spectrin, and they are consequently unable to alter erythrocyte morphology. HDL do prevent the LDL-induced activation of membrane phosphatase. The inhibitory capacity of HDL is observed over the range of LDL : HDL (w/w) which exists in the plasma of normolipemic humans.

Introduction

A variety of cells grown in culture have the ability to degrade plasma low density lipoproteins (LDL) [1—5]. This process involves binding of LDL to the

* To whom reprint requests should be addressed.

Abbreviations: VLDL very low density lipoproteins (*d* 1.006); LDL, low density lipoproteins (*d* 1.019—1.063); HDL, high density lipoproteins (*d* 1.063—1.21) [11,21].

cell surface, internalization via endosomes, and transfer of the lipoprotein into lysosomes following endosome-lysosome fusion [3,6,7]. Thus, in experiments measuring the response of cultured cells to added lipoproteins, it is difficult to distinguish lipoprotein-induced alterations of the cell surface from effects due to cellular uptake and degradation of the lipoproteins. To investigate the effects of binding of lipoproteins to the cell surface, we have employed freshly isolated mature human erythrocytes, a cell which does not internalize lipoproteins.

Various lipoproteins do alter membrane function. VLDL activate the adenylate cyclase system of plasma membranes isolated from human and rat adipocytes [8]. Shore and Shore observed that LDL promote agglutination of erythrocytes by concanavalin A [9] and activate a Mg^{2+}-ATPase in isolated erythrocyte membrane ghosts [10]. In the preceding manuscript [11], we describe the effect of LDL on the morphology of intact human erythrocytes; LDL induce a time-dependent transformation of erythrocytes from biconcave discs to spherocytes. The LDL-induced alteration of erythrocyte morphology occurs without concomitant leakage of hemoglobin from the cell. The alteration is also not due to depletion of intracellular ATP or to a redistribution of the major lipids of the erythrocyte membrane.

In this paper, we report the influence of LDL on the phosphorylation-dephosphorylation of membrane proteins and on the activity of the membrane-associated enzyme, acetylcholinesterase. The rationale for studing the former system is based on recent reports that phosphorylation and dephosphorylation of membrane proteins regulate erythrocyte morphology [12—17]: the spectrin complex, which may be responsible for the contractile activity associated with the erythrocyte membrane, is phosphorylated in biconcave discs and is dephosphorylated when the cells are crenated [12—15]. Furthermore, conditions which promote the discocyte-spherocyte transformation appear to decrease the function of acetylcholinesterase [18]. Inhibition of the enzyme by F^- depends on the lipid composition and fluidity of the membrane [19], suggesting that it is a sensitive indicator of membrane alterations.

Methods

Isolation of erythrocytes. Human blood was obtained from healthy male donors and was used immediately without storage. Samples were drawn into vacutainer tubes containing EDTA anticoagulant solution. The plasma and buffy coat were removed by aspiration following centrifugation. To remove residual white blood cells, the erythrocytes were further purified by the column chromatographic method of Poon and Simon [20]. The erythrocytes were reisolated by centrifugation and washed three times with 150 mM NaCl.

Isolation and characterization of lipoproteins. Lipoproteins were isolated from freshly collected human plasma by sequential ultracentrifugal flotation in KBr in the presence of EDTA [21]. Blood donors had fasted 12 h. The purity of each lipoprotein fraction was assessed by electrophoresis on agarose (1%), and by immunochemical analysis using antibodies to purified lipoproteins LDL and HDL and to selected apoproteins (AI, AII, B). The lipid composition (phospholipid, triglyceride, cholesterol, cholesteryl esters) of each lipoprotein

class was within the normal range [11,22]. Lipoprotein samples were dialyzed extensively against 150 mM NaCl prior to use.

Phosphorylation of membrane proteins in intact erythrocytes. Washed erythrocytes were resuspended to a 10% hematocrit. The volume of the typical reaction mixture was 10 ml with final concentrations as follows: 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 20 mM KCl, 5 mM glucose, 1 μM adenine, and 600 μCi of ^{32}P as $Na_2H^{32}PO_4$. When added, LDL were 2.5 mg/ml and HDL, 4.5 mg/ml. The concentrations of added lipoproteins are given as mg/ml lipoprotein. Following incubation for 3 h at 37° C, erythrocytes were isolated by centrifugation and were washed three times in 150 mM NaCl. Washed erythrocytes were lysed and membrane ghosts prepared according to the method of Dodge et al. [23]. Membrane proteins were subsequently precipitated with cold 10% trichloroacetic acid and then resuspended in a minimum volume of 1 M NaOH. The samples were diluted with water and the radioactivity determined by liquid scintillation in Aquasol (New England Nuclear).

Identification of labeled polypeptides. Polyacrylamide gel electrophoresis of membrane ghosts was performed in 6% acrylamide containing 0.16% *N,N'*-methylene-bis-acrylamide and 0.2% sodium dodecyl sulfate (SDS) as described by Fairbanks et al. [24]. Gels were sliced into 1-mm fractions and each slice was dissolved in 1 ml 30% H_2O_2. The radioactivity of each fraction was determined by liquid scintillation.

Phosphatase activity. To measure phosphatase activity of erythrocyte ghosts, *p*-nitrophenyl phosphate was employed [25,26]. A volume of freshly prepared membrane ghosts equivalent to 0.8 ml original packed cells was incubated at 37° C in 10 ml of 20 mM Tris-HCl (pH 7.4) containing 10 μM ATP as specified, 5 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate, and 2.5 mg/ml LDL and/or 4.5 mg/ml HDL when specified. At the end of incubation, a 1 ml aliquot was removed and the reaction was stopped by the addition of 0.2 ml 33% trichloroacetic acid. Following centrifugation to remove precipitated membranes and lipoproteins, 1 ml of the supernatant was added to 2 ml 0.2 M NaOH and the concentration of *p*-nitrophenol was determined spectrophotometrically at 418 nm. The maximum hydrolysis of *p*-nitrophenyl phosphate after a 3 h incubation was less than 20% of the initial amount of substrate present in the assay medium.

Protein kinase activity. Protein kinase activity of erythrocyte membrane ghosts was determined by measuring the phosphorylation of casein as described by Hosey and Tao [27]. Membrane ghosts were incubated in 20 mM Tris-HCl (pH 7.4) for 3 h at 37° C. LDL and HDL were added to a final concentration of 2.5 and 4.5 mg/ml, as specified. An equivalent volume of 150 mM NaCl was added to the control experiment. At the end of incubation, 0.1 ml of the suspension was added to an equal volume of a solution containing 100 μM [γ-^{32}P]-ATP and 4 mg/ml casein in 20 mM Tris-HCl (pH 7.4). After incubation for an additional 30 min, the reaction was terminated by the addition of 15 μl of 12 mg/ml bovine serum albumin and 2 ml 33% trichloracetic acid. The protein precipitate was collected on glass fiber discs (Whatman GF/C, 24 mm diameter) and washed exhaustively with 5% trichloroacetic acid; protein-bound ^{32}P was measured by liquid scintillation in Aquasol.

Dephosphorylation of endogenous membrane proteins. Erythrocyte ghosts were preincubated with [γ-^{32}P]ATP (approx. $7.5 \cdot 10^5$ cpm ^{32}P per mg protein) for 1 h at 37°C in 20 mM Tris-HCl (pH 7.4). Ghosts were washed exhaustively to remove residual ATP and were subsequently incubated in 20 mM Tris-HCl (pH 7.4) for 2 h in the presence or absence of 2.5 mg/ml LDL. At the end of incubation, membrane ghosts were washed and proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 5%. The acid-insoluble fraction was redissolved in a minimal volume of 1 M NaOH and radioactivity was determined as described above.

Erythrocyte acetylcholinesterase. Erythrocyte ghosts were incubated with 2.5 mg/ml LDL and/or 4.5 mg/ml HDL for 3 h at 37°C as described above. At the end of incubation, 0.1 ml ghost suspension was added to 2.9 ml 20 mM sodium phosphate (pH 8.0) containing 0.5 mM acetylthiocholine, 1 mM dithiobisnitrobenzoate, 1 mM $MgCl_2$, and NaF at the indicated concentration. The activity of acetylcholinesterase was determined by the colorimetric method of Ellman et al. [28]. The Hill coefficient (n) for F^- inhibition of acetylcholinesterase activity was determined graphically using the equation $\log[v/(V_0 - v)] = \log K - n \log[F^-]$ where V_0 is the velocity in the absence of F^-; v, the velocity in the presence of F^-.

Analytical procedures. Protein concentration was determined in 1% SDS by a modified method of Lowry et al. [29] using bovine serum albumin as the standard. Total triglyceride was determined enzymatically [30] using the Triglyceride Test Combinations Kit (Boehringer Mannheim). Total phospholipid was measured as phosphorus by the method of Bartlett [31]; and cholesterol and cholesteryl esters were determined by the method of Roeschlau et al. [32] using the Cholesterol Test Combination Kit (Boehringer Mannheim).

Results

When intact erythrocytes are incubated with [^{32}P]phosphate, radioactivity is progressively incorporated into the membrane protein fraction as illustrated in Fig. 1. However, when erythrocytes are incubated in the presence of LDL, ^{32}P uptake is linear during the initial 2 h of incubation only, and after 4 h there is a 40% decrease in net membrane protein phosphorylation relative to the control. It is unlikely that this decrease is due to a reduction in available [γ-^{32}P]ATP since the intracellular ATP level and the intracellular level of [^{32}P]-phosphate are unaffected by incubation with the lipoproteins [11]. The 2 h time required for LDL to influence erythrocyte membrane phosphorylation coincides with the onset of disc → sphere morphological transformation [11]. Phosphorylation-dephosphorylation is not perturbed when erythrocytes are incubated with HDL (Fig. 1) or with a combination of equimolar quantities of LDL and HDL (data not shown).

Singularly, the observed decrease in net [^{32}P]phosphate content of 40% is not particularly noteworthy. To determine if the decrease in LDL-treated erythrocytes could be attributed to a decrease in phosphorylation of a specific membrane protein, the proteins were partially resolved by SDS-polyacrylamide gel electrophoresis and analyzed for the presence of [^{32}P]phosphate (Fig. 2). Erythrocyte ghosts from cells which are incubated in the absence of added

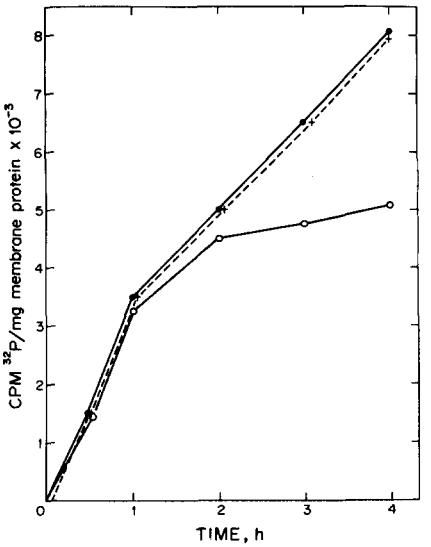


Fig. 1. Phosphorylation of erythrocyte membrane proteins in intact cells. Human erythrocytes were incubated at 37°C in 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 20 mM KCl, 10 mM glucose, 1 μM adenine, 600 μCi of ^{32}P as $Na_2H^{32}PO_4$, in the absence of lipoproteins (●———●), in the presence of 2.5 mg/ml LDL (○———○), or in the presence of 0.25 mg/ml HDL (X- - - - -X). The error limits are ±5% (quadruplicate determinations).

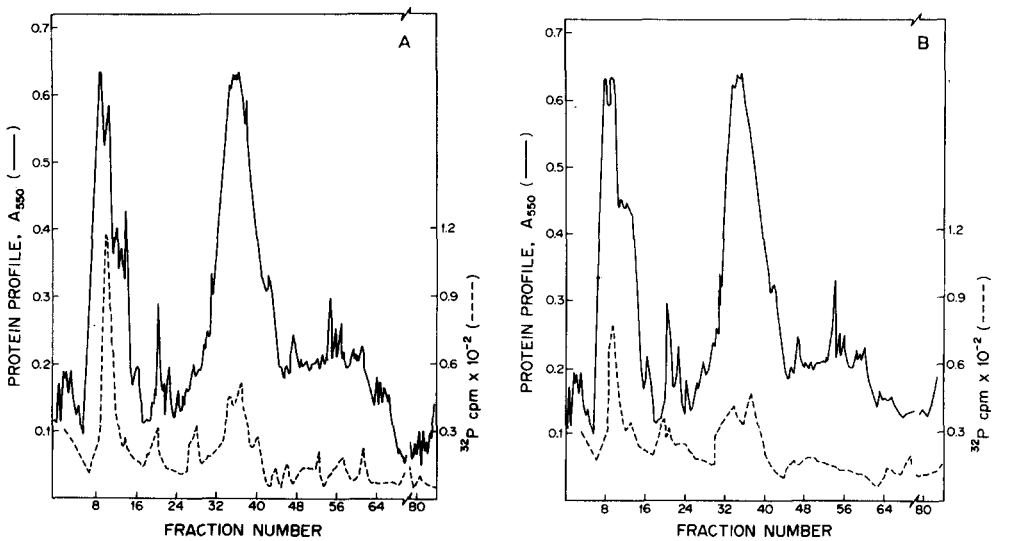
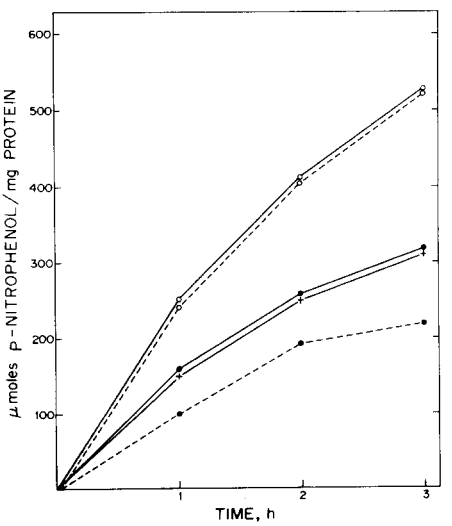


Fig. 2. SDS gel electrophoresis of membrane proteins. Intact cells were incubated for 4 h as described in Fig. 1 with (A) no added lipoproteins; or (B) 2.5 mg/ml LDL. Membrane ghosts were prepared and polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al. [24]. 50 μg membrane protein was applied to each gel. Gels were stained with Coomassie Blue and destained in 10% acetic acid. Protein profiles were determined spectrophotometrically at 550 nm. Gels were sliced into 1-mm fractions, dissolved in 1 ml 30% H_2O_2 and counted to determine radioactivity. Band classification: I, fraction 8; II, fraction 10; III, fractions 32—42. Bands I and II are spectrin bands [24]. The error limits are ±5% (triplicate determinations).

lipoproteins contain radioactive phosphate in components II, III and in other minor protein fractions (Fig. 2A). The LDL-induced decrease in ^{32}P incorporation is not due to a loss of membrane proteins (compare Fig. 2A with Fig. 2B), but is attributed entirely to a specific decrease in the phosphorylation of spectrin component II (Fig. 2B).

To investigate the effect of LDL on the phosphorylation and dephosphorylation independently, experiments were performed using isolated membrane ghosts and exogenous enzyme substrates. Membrane phosphatase activities were measured by monitoring the hydrolysis of *p*-nitrophenyl phosphate. When erythrocyte ghosts are incubated with *p*-nitrophenyl phosphate, a progressive hydrolysis of the substrate to *p*-nitrophenol occurs as shown in Fig. 3. However, the addition of LDL to the incubation medium substantially increases the rate of hydrolysis. Moreover, activation of phosphatase activity increases with increasing concentration of LDL (Fig. 4); activation is not maximal even at LDL concentrations of 4 mg/ml. When LDL is incubated with *p*-nitrophenyl phosphate in the absence of erythrocyte ghosts, no hydrolysis occurs. Therefore, the increased hydrolysis induced by LDL must be attributed to an increase of phosphatase activity in LDL-incubated erythrocyte ghosts. On the other hand, when erythrocyte ghosts are incubated with HDL, phosphatase activity is unaltered (Figs. 3 and 4); HDL added at equimolar amounts does, however, prevent the LDL-induced activation of *p*-nitrophenyl phosphate phosphatase. The hydrolysis of *p*-nitrophenyl phosphate by erythrocyte ghosts is partially inhibited by the addition of 10 μM ATP as indicated in Fig. 3. Phos-



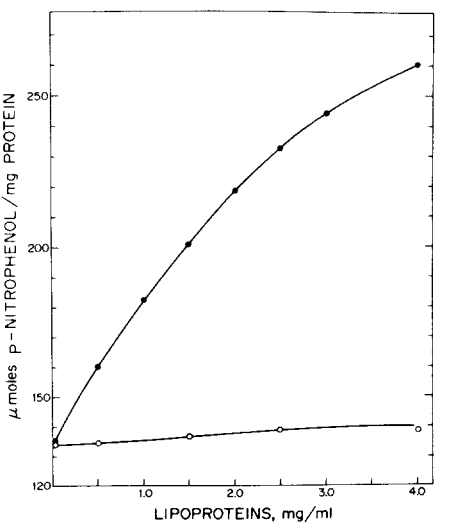


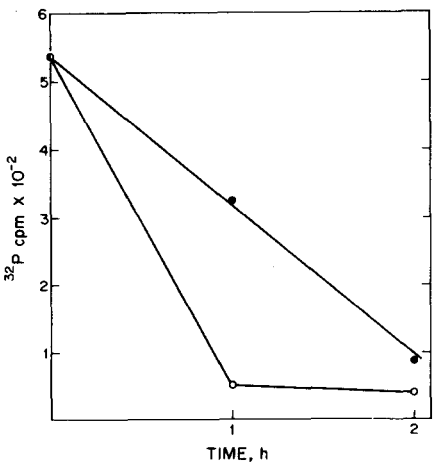
Fig. 3. Phosphatase activity of isolated erythrocyte membranes. Human erythrocyte ghosts were incubated at 37° C in 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, containing 5 mM *p*-nitrophenyl phosphate and either: no further additions (●———●); 10 μm ATP (●- - - - -●); 2.5 mg/ml LDL (○———○); 2.5 mg/ml LDL plus 10 μM ATP (○- - - - -○); 4.5 mg/ml HDL (X———X).

Fig. 4. Dependence on lipoprotein concentration of activation of erythrocyte membrane phosphatases. Human erythrocyte ghosts were incubated with LDL (●———●) or HDL (○———○) for 1 h at 37° C and the amount of *p*-nitrophenol produced was determined spectrophotometrically.

phatase activity in the presence of LDL is insensitive to inhibition by ATP. LDL also accelerate the dephosphorylation of phosphorylated erythrocyte membrane proteins. As illustrated in Fig. 5, dephosphorylation catalyzed by membrane-associated phosphoprotein phosphatase occurs rapidly in the presence of 2.5 mg/ml LDL relative to the control.

Another mechanism which might account for the decreased incorporation of phosphate into membrane proteins in the presence of LDL is a decrease in the activity of cyclic AMP-independent protein kinases associated with the human erythrocyte membrane. When incubated in the presence of [γ-^{32}P]ATP, ^{32}P is transferred to the soluble protein fraction which contains casein, an appropriate exogenous substrate. However, the amount of ^{32}P incorporation into casein is not influenced by preincubating erythrocyte ghosts with either LDL or HDL: $3.12 \cdot 10^3$ (±3%) cpm of ^{32}P are incorporated per mg casein in the presence or absence of added lipoproteins. Thus, cyclic AMP-independent protein kinase activities are not influenced by the LDL-erythrocyte interaction.

Erythrocyte acetylcholinesterase is altered in response to the addition of LDL: although the basal activity of the enzyme is identical in the presence and absence of LDL, its sensitivity to inhibition by F^- is diminished. When erythrocyte ghosts are incubated with F^-, acetylcholinesterase activity decreases as F^- concentration increases as illustrated in Fig. 6; the Hill



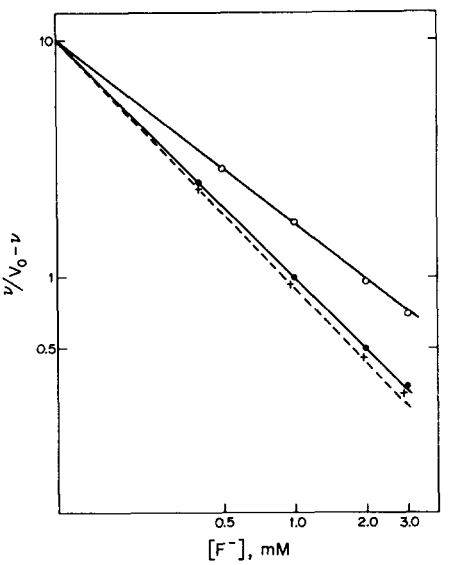


Fig. 5. Dephosphorylation of endogenous membrane proteins. Erythrocyte membrane ghosts were preincubated with [γ-^{32}P]ATP for 1 h at 37°C in 20 mM Tris-HCl (pH 7.4). The ghosts were washed exhaustively and incubated in 20 mM Tris-HCl (pH 7.4) as indicated in the absence of lipoprotein (●——●), or in the presence of 2.5 mg/ml LDL (○——○). ^{32}P counts in the protein fraction were determined after trichloroacetic acid precipitation.

Fig. 6. F^- inhibition of erythrocyte acetylcholinesterase activity. Erythrocyte ghosts were incubated for 3 h at 37°C in 20 mM sodium phosphate (pH 8.0) containing 0.5 mM acetylthiocholine, 1 mM dithiobisnitrobenzoate, 1 mM $MgCl_2$, and NaF at the indicated concentrations. The incubation was performed without lipoproteins (●——●); with 2.5 mg/ml LDL (○——○), or with 4.5 mg/ml HDL (X- - - - -X). The Hill coefficient in each case was determined from the slope of the plot. The error limits are <±1%.

coefficient for inhibition is 1.0. When the ghosts are preincubated with LDL for 30 min prior to the addition of F^-, the Hill coefficient for inhibition decreases to 0.8. On the other hand, when the ghosts are preincubated with HDL, the Hill coefficient for F^- inhibition is identical to that obtained in the absence of lipoproteins.

Discussion

It is now well documented that LDL are endocytized by nucleated cells and, as a result, regulate cholesterol biosynthesis [33]. However, little is known about the role of LDL in the function of a cell type which lacks the ability to internalize and degrade lipoproteins. Data presented herein demonstrate that the addition of LDL to a suspension of intact human erythrocytes results in a decrese in the phosphate content of spectrin, a major erythrocyte cytoskeletal protein. The time required for LDL to reduce the level of spectrin phosphate, 2 h at 37°C, coincides with the onset of the LDL-induced disc → crenated sphere transformation reported previously [11], a fact which supports the hypothesis that phosphorylation of this membrane-associated protein is critical for erythrocyte morphology [13—15]. Our data are also consistent with recent reports that spectrin phosphorylation governs the interaction of spectrin with actin [34] and may regulate the structural state of the spectrin-actin complex on the cytoplasmic surface of the membrane [15]. The extent of spectrin phosphorylation is uncertain. Moreover, it is not known whether phosphorylation of a certain spectrin residue correlates with a specific property of this cytoskeletal protein. Harris et al. [35] detect four phosphorylated spectrin sites, two of which have significant turnover rates. At present, we have no direct evidence indicating that LDL elicit dephosphorylation of specific spectrin sites. It has been adequately demonstrated [13,36,37] that decreases of 40—60% of the phosphate content of spectrin are consistently associated with abnormal erythrocyte morphology. Therefore, it is of particular interest that LDL cause a net reduction of 40% in the phosphate content of spectrin.

The simplest mechanism to account for the decreased level of phosphorylation of spectrin when erythrocytes are incubated with LDL is a reduction of intracellular ATP. However, LDL do not influence the intracellular concentration of ATP [11]. Since the phosphate content of spectrin depends on the balance between activities of membrane-associated cyclic AMP-independent protein kinases and phosphoprotein phosphatases [16], we have pursued the possibility that LDL specifically inhibit phosphorylation and/or stimulate dephosphorylation of spectrin. LDL do not influence the activities of the membrane-bound protein kinases. However, membrane-associated phosphatases are stimulated 2-fold and greater by LDL. The extent of phosphatase activation depends on the concentration of LDL. Phosphatase activation by LDL was demonstrated by two methods: hydrolysis of *p*-nitrophenyl phosphate and hydrolysis of phosphorylated membrane proteins. Significantly, HDL do not accelerate dephosphorylation of *p*-nitrophenyl phosphate nor do they induce dephosphorylation of spectrin or alteration of erythrocyte morphology [11]. HDL do prevent the LDL-induced alterations, apparently by competitively inhibiting the interaction of LDL with the erythrocyte membrane [11].

The detailed sequence of events resulting in phosphatase activation by LDL is not evident from results of the present studies. Activation is not due to major changes in the lipid composition of the cell membrane as a result of lipid exchange/transfer with LDL [11]. Detailed information describing the properties of membrane-bound phosphoprotein phosphatases is lacking. There is some evidence which suggests that phosphatase activity may be regulated by a cyclic AMP-dependent conversion of an inactive enzyme-inhibitor complex to an active enzyme [38]. However, since the mature erythrocyte lacks a functional adenylate cyclase system [39], it would seem that analogous activation of the erythrocyte enzyme must be controlled by other signals. Possible signals may be provided by the ability of LDL to: (1) modify the lipid composition in a critical region of the membrane; (2) modify the bulk or localized viscosity of the membrane; and/or (3) perturb the concentration of intracellular ions. With respect to point 2 we note that LDL alter the response of a membrane-associated enzyme acetylcholinestersase to inhibition by F^- (Fig. 6) in a manner indicative of an LDL-elicited decrease in membrane fluidity since the Hill coefficient for F^- inhibition decreases [19]. However, it is equally possible that LDL alter the accessibility of the enzyme to the inhibitor. With respect to point 3, LDL do not induce a permeability defect for Na^+ in the cell membrane such as that introduced by the addition of sonicated phosphatidylcholine vesicles to intact erythrocytes [40]. The concentration of Na^+ in intact cells incubated for 4 h with LDL is identical to the value obtained for control cells incubated without lipoproteins (Hui, D.Y. and Harmony, J.A.K., unpublished data). Studies are currently underway to determine whether LDL influence the intracellular concentration of other ions.

Finally, we wish to point out that our findings are very similar to those of Loyter et al. [41] who report that Sendai viruses, which induce erythrocyte lysis and fusion, also cause dephosphorylation of erythrocyte membrane proteins. It is significant in their studies [41] that phosphoprotein phosphatase activity is not stimulated by non-ionic detergents or by the hemolytic toxin prymnesin, lending support for our previous conclusion [11] that the LDL-induced alterations are not the result of a detergent-like disruption of the cell membrane.

In conclusion, this is the first report that plasma lipoproteins, in particular the LDL, induce dephosphorylation of membrane-associated proteins. The significance of this finding is augmented by the fact that the target for dephosphorylation is spectrin, a cytoskeletal protein which specifies cell shape [13–15] and the distribution of receptors at the cell surface [42,43]. Spectrin has so far been identified in the erythrocyte only, although high molecular weight cytoskeletal proteins which interact with actin are postulated to exist in other cell types [44]. Our observations that lipoproteins present in the high density class are not able to stimulate dephosphorylation of erythrocyte spectrin or to alter erythrocyte morphology, but that HDL prevent LDL-induced alterations are of considerable interest since they relate to recent suggestions that HDL is a protective factor against the development of cardiovascular disease [6]. The inhibitory capacity of HDL is observed over the range of LDL : HDL which exists in the plasma of normolipemic humans. Whether the ratio of LDL to HDL is critical for erythrocyte morphology and physiology remains to be determined.

Acknowledgements

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