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# Action of Phospholipase D on Human Serum Low Density Lipoprotein (40443)

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Phospholipases have been used in the past to study the role of phospholipids in the structure and stability of biological membranes and lipoproteins. Of the different phospholipases, types A or C are used more commonly and may ultimately remove the phospholipid from the parent structure either as lysolecithin or as a diglyceride. However the product of the action of phospholipase D is phosphatidic acid which, being highly surface active presumably remains at the original site of the phospholipid (1). It may therefore be expected that the degree of hydrolysis of the phospholipid of a lipoprotein by the latter enzyme would give a measure of the phospholipid groups exposed on the surface of the lipoprotein. In this work we report the action of phospholipase D of serum low density lipoprotein (LDL) from normocholesteremic and hypercholesteremic individuals (LDL-N and LDL-C). We have already reported (2), that the surface charge characteristics of LDL isolated from these two groups are different. Our objective in this work was to investigate to what extent phospholipase D may be used to probe the surface phospholipid groups of these lipoproteins.

*Materials and methods. Human low density lipoprotein* ($S_f$ 0–20): EDTA plasma was obtained from fasting (16 hr) human subjects and LDL was prepared from the individual plasma samples by sequential ultracentrifugation. The subjects were either normocholesteremic healthy individuals (called normal hereafter) or hypercholesteremic Fredrickson type II patients. The samples from patients were supplied by Dr. D. Berksen of St. Joseph Hospital, Chicago. They were type II on the basis of plasma cholesterol (350–900 mg/dl) and triglyceride (135–260 mg/dl), as well as by agarose gel electrophoresis. The ultracentrifugation method employed was similar to one described by Hatch and Lees (3). The details of the separation procedure using rotor 65 have been given elsewhere (4). The samples of LDL obtained by ultracentrifugation were dialyzed exhaustively against 0.15 *M* NaCl to remove bromide used for ultracentrifugation. The samples were tested for contamination by other proteins by immunoelectrophoresis using anti-human serum, as well as by agarose gel electrophoresis followed by Coomassie blue staining. All the samples used in this work gave single bands in both the tests.

*Phospholipase D* (EC 3.14.4), prepared from cabbage (Lot No. 52C-2350, P-7758) was purchased from Sigma Chemical Company, St. Louis, MO. Its activity was assayed using a dispersion of egg lecithin as substrate according to the method Davidson and Long (5). Contaminating proteolytic activity was found to be negligible by the method of Anson (6).

*Antiserum.* Anti-human serum (horse) for immunoelectrophoresis was obtained from Behring Diagnostics, Woodbury, New York. All other chemicals were A.R. grade.

*Incubation of LDL with Phospholipase D.* Incubations were done in presence and absence of ether as activator. The procedure was in general similar to that of Bruckdorfer and Green (7). For incubations done in presence of ether, 1 ml of LDL was mixed with 1 ml of Tris buffer, of pH 7.1 and ionic strength 0.1, saturated with diethyl ether. For incubations in absence of ether, the buffer was not equilibrated with ether. To the mixture $CaCl_2$ was added to a concentration of 0.12 *M* unless mentioned otherwise. 1.25 ml of enzyme solution (1.0% in buffer) was then added and the mixture was incubated in a stoppered 13.0 ml tube, in a Dubnoff shaker at 26°, for definite intervals of time. The rather large amount of enzyme was necessary for measurable activity in absence of ether. The rest of the procedure was similar to that of Davidson and Long (5). After the incubation, the enzymic reaction was stopped by adding 0.75 ml of (w/v) trichloroacetic acid



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(TCA). The mixture was shaken thoroughly after addition of 1 ml ether and 0.1 ml chloroform to make the system biphasic, and the tubes were centrifuged (2000 rpm, 20 min). The organic phase and the TCA-precipitate containing proteins and the phosphatidic acid were removed with a Pasteur pipette and discarded. The aqueous phase containing the choline product was filtered and the clear filtrate was warmed on a water bath at 50°C, with Vortex agitation every thirty minutes to remove dissolved ether. Choline was determined in the ether-free filtrate according to the method of Shapiro (8).

*Electrophoresis.* Electrophoretic mobility of LDL-N before and after enzyme digestion, was determined in 0.6% agarose gel in Tris buffer (pH 7.4, I = 0.05). The details of the method have been described previously (4).

*Immunoelectrophoresis.* This was carried out in a Hyland agar gel immunoelectrophoresis sytem, using barbital buffer (pH = 8.6, I = 0.02). After ½, 2½ and 5 hr digestion of LDL with phospholipase D, a 10 μl sample of the digested lipoprotein was transferred to a well on the plate and the electrophoresis was run immediately. The plates were developed with anti-human serum.

*Results and discussion.* It may be emphasized that this work concerns the use of phospholipase D to probe the surface of native LDL; the incubation conditions had, therefore, to be adjusted as close to physiological pH, and as low a calcium concentration as possible so as not to denature the LDL; omission of ether was also necessary for that purpose and the experiments were necessarily done under suboptimal conditions. Optimal conditions with ether and higher $CaCl_2$ were used only to determine the total lecithin of the system. Secondly, the amounts of LDL that could be isolated from the available plasma samples were small, and the experiments were done at relatively low substrate to enzyme ratios.

Davidson and Long (5) and Dawson and co-workers (9, 10) have characterized the enzyme properties of phospholipase D. It has been shown that the enzyme hydrolyzes lecithin as well as phosphatidyl ethanolamine dispersions with the liberation of choline and ethanolamine respectively. The optimum pH with both of these substrates was around 5.6 (9). Although there is a possibility of the shift of the optimum towards pH 7 when considerable accumulation of phosphatidic acid occurs (10), phosphatidyl ethanolamine constitutes only 3–4% of the phospholipid of LDL compared to 62–68% lecithin, and the base determined by the method of Shapiro (8) may be taken to be choline. Under the conditions of our experiments shingomyelin (used as a dispersion) was not hydrolysed by the cabbage enzyme. Reaction with lysolecthin, a very minor constitutent of LDL, is also known to be very slow. The enzyme action may therefore be assumed to be directed primarily toward the lecithin of LDL.

The initial series of experiments were aimed at establishment of conditions under which probing of surface lecithin of LDL by phospholipase D may be feasible. These were performed using LDL-N, unless otherwise mentioned. The effect of LDL concentration on the extent of hydrolysis showed saturation behavior typical of enzymes. LDL concentrations of about 10 mg/ml, as used in most of the later experiments, were much below the saturation level. Figure 1 shows the effect of time of incubation on the liberation of choline in presence and absence of ether. It shows that the enzyme hydrolysed LDL lecithin even in absence of ether, although to a less extent. The activating effect of ether on the action of phospholipase D is well known (5, 9). Bruckdorfer and Green (7) have shown that in the presence of half-saturated ether about 80% of LDL lecithin was hydrolyzed in 1 hr with the production of choline and phosphatidic acid, the latter remaining with

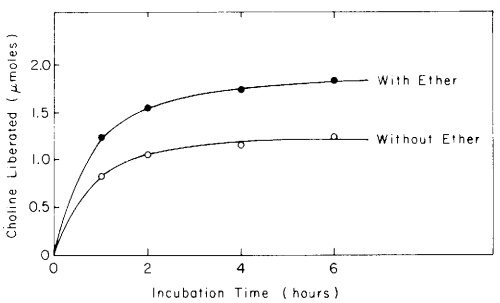


FIG. 1. The effect of time of incubation of LDL with phospholipase D. The incubations were done in Tris buffer (pH 7.1) with and without ether in presence of 0.12 *M* $CaCl_2$. The LDL concentration used was 9.6 mg/ml.

the LDL particle. However, since ether may potentially alter the lipoprotein structure, it was not used in the experiments aimed at probing the structure of native LDL. In the absence of ether, the reaction rate increased with an increase in the concentration of $Ca^{2+}$ and reached a maximum around 0.25 *M*; at 0.125 *M* $CaCl_2$ the reaction was 86% of the maximum and has been used in some experiments. The optimum concentration of $Ca^{2+}$ for lecithin was 0.039 *M* at pH 5.8 (9) but a much higher concentration was necessary at a higher pH (10). The higher concentration of $Ca^{2+}$ required here may be due to the net negative charge of LDL at the pH of the experiments (2).

The difference in mobility ($\Delta\mu$) between the enzyme modified LDL and the control LDL has been plotted against the time of incubation in Fig. 2. The surface charge of LDL became increasingly negative with the time of digestion, consistent with a progressive accumulation of phosphatidic acid during hydrolysis of lecithin by phospholipase D.

Immunoelectrophoretic pattern (precipitin line for LDL) for LDL-N and LDL-C remained the same, after 5½ hr reaction with the enzyme in absence of ether, as for the untreated lipoproteins. This, as well as the sharp electrophoretic patterns of the enzyme treated LDL, showed the presence of intact LDL particles without appreciable loss of immunoreactivity. The action of any contaminating proteolytic enzyme or phospholipase C, to the extent it is expected to lower the immunoreactivity or the stability of LDL (7) respectively, may therefore be considered negligible in our experimental system.

It therefore appears that probing of the surface lecithin groups of native LDL by phospholipase D is possible at pH ~ 7 in absence of ether.

The kinetics of hydrolysis of LDL-N and LDL-C, isolated simultaneously from fresh plasma were then studied for three pairs of samples. At the same concentration, LDL-C consistently showed a higher extent of hydrolysis than LDL-N, especially in the initial stage of the reaction. For each of these samples an additional aliquot of LDL was digested with phospholipase D for 6 hr in presence of ether and 0.25 *M* $CaCl_2$. The choline liberated in this procedure was found to approach within experimental error the lecithin present in the sample of LDL for both of LDL-N and LDL-C and was used to calculate the percent of lecithin hydrolysed at different intervals of time. Semilog plots of these values are shown in Fig. 3.

The net course of an enzyme reaction may, in principle, be described by an integrated equation, which reduces to that of a first order reaction at suboptimal substrate concentration (11). Furthermore, Aggerbeck and Scanu (12) observed that the hydrolysis of LDL by phospholipase A followed first order kinetics. Semilog (first order) plot of our data was linear for LDL-N (Fig. 3) during the time period of study; extrapolation of the line to zero time however gave a substrate level

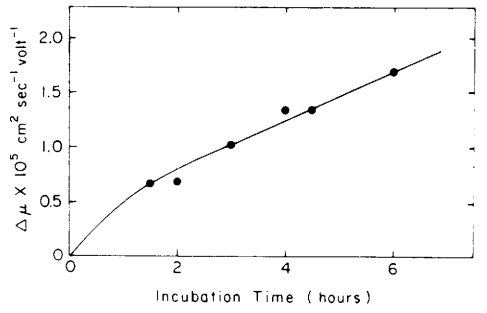


FIG. 2. The difference in electrophoretic mobility ($\Delta\mu$) between the enzyme modified LDL and the control LDL plotted against the time of enzyme treatment. The electrophoretic mobility was determined in agarose gel (0.6%) at pH 6.8 and ionic strength 0.05.

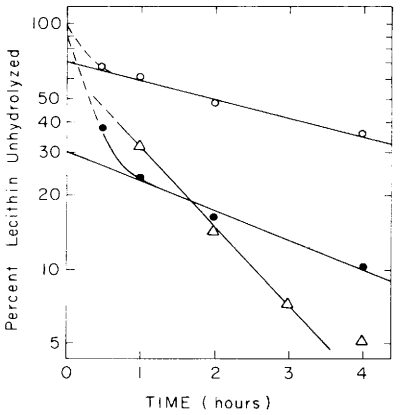


FIG. 3. Semilog plots of the hydrolysis of LDL-N and LDL-C in presence of 0.12 *M* $CaCl_2$: (○——○) LDL-N, (●——●) LDL-C, in absence of ether. (△——△) LDL-N with ether.

much lower than 100%, being 60–70% at a $CaCl_2$ concentration of 0.12 *M* in absence of ether. Under these conditions the lecithin moieties of LDL were not behaving as a single pool and part of the LDL lecithin was more susceptible to the enzyme action than the rest of it. The effect of ether (Fig. 3) appears to be in making the slower pool more susceptible to the action of phospholipase D. Further work using well defined model systems will be needed to prove this point. It is interesting to note that a previous structure proposed for LDL (13) suggested the existence of a phospholipid bilayer in the LDL molecule with a part of the phospholipid buried inside.

The semilog plot for LDL-C (Fig. 3) shows that the slope of the linear region was, within experimental error, the same as for LDL-N, but the intercept at zero time was lower (30–40% of the total substrate). This suggests that the fraction of lecithin more susceptible to enzyme hydrolysis was higher in LDL-C than in LDL-N. The observed difference is not related to the lecithin content of the molecules *per se*, since the percentage of lecithin in the total weight of LDL-C is in general slightly lower, 12% compared to 14% in LDL-N as reported by Mills and Taylaur (14), who reviewed reported differences in properties like dielectric decrement and hydration (15) between LDL-N and LDL-C. After detailed examination of the compositional difference between the two, Mills and Taylaur concluded that such differences for individual components are not sufficient in explaining the difference in the physical properties and suggested that the structural arrangements of components are different in these two species of molecules. Our observations are in keeping with this suggestion.

*Summary.* Phospholipase D was used to investigate the lecithin groups on the surface of serum low-density lipoprotein (LDL), isolated from normo- as well as hypercholesteremic individuals. The rate of liberation of choline from lipoprotein lecithin was studied in presence and absence of ether and in different concentrations of $CaCl_2$. The kinetics of the digestion in absence of ether suggested that the lecithin groups of LDL are not equally susceptible to enzymic hydrolysis. The effect of ether was to increase the susceptibility of the slowly digesting group of lecithin. Compared to LDL isolated from normal controls, LDL from hypercholesteremic individuals seemed to have a much larger fraction of lecithin that was digested at a faster rate. Immunological activity is retained in normal as well as hypercholesteremic LDL, even after hydrolysis of 75% of the total lecithin.

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