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Aggregation of dimyristoylphosphatidylglycerol liposomes by human plasma low density lipoprotein

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Abstract

Turbidity (absorbance at 470 nm) measurements revealed human serum low density lipoprotein (LDL) to cause, within a few minutes and at physiological pH and [NaCl], the aggregation of liquid crystalline large unilamellar liposomes (LUVs) of dimyristoylphosphatidylglycerol (DMPG). No evidence for concomitant lipid or aqueous contents mixing was obtained with fluorescent assays for these processes, in keeping with the lack of fusion of LUVs. Involvement of apoB is implicated by the finding that tryptic digestion of LDL abrogates its ability to cause aggregation. Aggregation is not caused by VLDL, HDL_2, or HDL_3. Interestingly, also oxidised LDL failed to aggregate DMPG vesicles. Aggregation of DMPG LUVs by LDL did depend on the ionic strength of the medium as well as on the phase state of the lipid. More specifically, below the main transition temperature T_m maximal aggregation was seen in the presence of 25–100 mM NaCl, whereas slightly higher (up to 150 mM) [NaCl] were required when $T > T_m$. Aggregation due to LDL was also observed for dimyristoylphosphatidylserine as well as for dipalmitoylphosphatidylglycerol LUVs, whereas liposomes composed of either unsaturated acidic phospholipids or different phosphatidylcholines were not aggregated. Involvement of electrostatic attraction between the acidic phosphate of DMPG and cationic residues in apoB is suggested by the finding that increasing the content of dimyristoylphosphatidylcholine (DMPC) in DMPG liposomes reduced their aggregation and at $X_{DMPC} = 0.50$ no response was evident. Notably, increasing the mole fraction of 1-palmitoyl-2-oleyl-PG (POPG) in DMPG LUVs progressively reduced their aggregation by LDL and at $X_{POPG} = 0.50$ there was complete inhibition. The latter effect of POPG is likely to be due to augmented hydration of the unsaturated lipid constituting a barrier for the contact between apoB and the vesicle surface. In keeping with this view, the presence of the strongly hygroscopic polymer, poly(ethylene glycol) at 1% (by weight) enhanced the aggregation and could partly reverse the inhibition by POPG.

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulphonate; BSA, bovine serum albumin; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine; DPPF, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol-*N*-thiocarbamoyl-5-fluorescein; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DPX, *p*-xylylenebis(pyridinium bromide); triethylammonium salt; DSC, differential scanning calorimetry; HDL, high density lipoprotein; LDL, low density lipoprotein; LUV, large unilamellar vesicles; MLVs, multilamellar vesicles; PEG, (polyethylene)glycol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PMSF, phenylmethanesulphonylfluoride; POPG, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoglycerol; PPDPG, 1-palmitoyl-2-[10-(pyren-1-yl)] decanoyl-*sn*-glycero-3-phosphoglycerol; PS, phosphatidylserine; TES, *N*-[tris-(hydroxymethyl)methyl]-2-aminoethanesulphonic acid; TLC, thin layer chromatography; VLDL, very low density lipoprotein

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1. Introduction

One of the most common causes of death in industrial countries is occlusion of blood vessels due to atherosclerosis, resulting from the formation of lipid plaques in the arterial walls. High blood levels of cholesterol correlate with atherosclerosis and the former is directly related to the contents of cholesterol and saturated fat in the diet. Atherosclerosis is, however, a multifactorial disease which also involves a strong genetic component [1].

Most of the plasma cholesterol is present in low density lipoproteins (LDL), an essential component of the plasma lipid transport system. The metabolism of LDL involves recognition of its protein moiety apolipoprotein B-100 by specific receptors, followed by endocytotic uptake. Cholesteryl esters of the internalised particles are thereafter hydrolysed in lysosomes and the liberated cholesterol and fatty acids reutilised by the cells [2–4]. In addition, oxidation of LDL has been shown to be an important determinant for its catabolic cellular processing [5], and to be associated with an enhanced risk for the development of atherosclerosis [6,7]. Lack of the LDL receptors causes the highly elevated levels of plasma LDL in the familial type-II hypercholesterolaemia [8–11], a disorder associated with an enhanced development of atherosclerotic lesions [6,12–14]. While this defect as well as epidemiological studies provide clear evidence for the importance of high plasma LDL concentrations in augmenting cholesterol deposition into the arterial intima the exact molecular level events of this process have remained unresolved. To this end, both smoking and high blood pressure represent additional, independent risk factors for atherosclerosis [15,16]. The molecular mechanism by which the latter two enhance cholesterol accumulation into atherosclerotic lesions are unknown.

Both extracellular and cellular pathways for the accumulation of LDL cholesterol into atheroma have been postulated and are both supported by findings from in vitro experiments, cell studies, as well as by histological data on diseased tissues. Smith [17] proposed atheroma formation to be an *extracellular* process, initiated by the binding of LDL to glycosaminoglycans, elastin, and proteoglycans [18–21]. Subsequently, this binding would be followed by the aggregation and fusion of LDL so as to result in the formation of lipid droplets [22] and the accumulation into the intima of deposits of cholesterol and cholesteryl esters, liposomes enriched in free cholesterol, and cholesteryl ester enriched lipoproteins [23,24]. Upon progression of the lesions, further accumulation of cholesterol into the atheroma core ensues, so as to finally result in their rupture [25]. When such rupture occurs in the coronary arteries, this further causes thrombus formation and heart attack [26].

The proposed *intracellular* pathway for atheroma formation involves the fusion of LDL particles after their binding to the negatively charged sulphated glycosaminoglycans of the arterial intima [27–29]. Thereafter, these aggregated and fused LDLs should be taken up by macrophages, which then become foam cells, characteristic to the intima affected by atherosclerotic lesions [30]. The above two mechanisms are not mutually exclusive and could both contribute to lipid accumulation into arterial walls. Interestingly, also vigorous mixing by vortexing (i.e. exposure to an excess of an air/water interface) causes the aggregation of LDL in vitro [31,32]. Enhanced uptake of such aggregated LDL by macrophages has been recently demonstrated [33]. This aggregation is prevented by HDL and possible linkage to the antiatherogenic effect of the latter lipoprotein was suggested [34].

Both lipid–lipid and lipid–protein interactions in LDL have been extensively investigated by a variety of techniques, including differential scanning calorimetry [35–37]. Likewise, interactions of LDL with various proteins and cell types have been studied [38–41]. While the association of cholesterol-containing liposomes with HDL and LDL has been reported [42–44] our knowledge on the interaction of LDL with lipid surfaces is still limited. Coprecipitation of cholesteryl ester/phosphatidylcholine microemulsions with LDL in vitro has been shown [45]. This complex formation could be prevented by apolipoprotein A-I, and did not depend on the unsaturation or saturation of the phospholipid. In another study [46], it

was shown that tryptic digestion of LDL abolished its coprecipitation with cholesteryl ester/phosphatidylcholine microemulsions.

Electrostatic interaction between LDL and negatively charged glycosaminoglycans is well established [27–29] and is likely to be mediated by positively charged clusters of lysine and arginine of apoB [47]. However, we are not aware of any studies on possible, similarly electrostatically mediated interactions between LDL and acidic phospholipids. A recent investigation revealed incubation of LDL with an excess of cardiolipin to result in an enhanced uptake of the lipoproteins by macrophages [48]. We demonstrate here that LDL, but not HDL or VLDL, causes, under proper conditions, an extensive aggregation of LUVs composed of saturated acidic phospholipids. Possible mechanism as well as physiological and pathological significance of the major findings of the study are briefly discussed.

2. Materials and methods

2.1. Materials

DMPG, DMPS, cholesterol, egg PG, POPG, PS (from bovine brain), and bovine pancreatic trypsin were from Sigma. DPPG was from Alexis (Läufelfingen, Switzerland), DMPC from Princeton Lipids (Princeton, NJ, USA), PPDPG from K&V Bioware (Espoo, Finland), and DPPF from Molecular Probes (Eugene, OR, USA). PEG (average molecular weight of 6000) was from Fluka. The purity of the above lipids was verified by thin-layer chromatography on silica gel-coated plates (Merck, Darmstadt, Germany) using chloroform/methanol/water/ammonia (65:20:2:2, by volume) as the solvent system. Examination of the plates after iodine staining and, when appropriate, upon UV-illumination revealed no impurities. For saturated phospholipids also differential scanning calorimetry (DSC) scans were performed using MLVs and gave endotherms with peak maxima and enthalpies identical to those in literature. Phospholipid concentrations were determined as organic phosphate by the method of Bartlett [49].

2.2. Isolation of plasma lipoproteins

Human VLDL (density fraction <1.006 g/ml), LDL ($d=1.019$–1.063 g/ml), total HDL ($d=1.063$–1.215 g/ml), HDL_2 ($d=1.063$–1.125 g/ml), and HDL_3 ($d=1.125$–1.215 g/ml) were isolated by sequential ultracentrifugation [50]. Comparison of LDL from single donors revealed essentially no differences. Accordingly, pooled plasma from healthy donors was used for lipoprotein isolation. For removal of KBr LDL was further subjected to gel filtration on a Sepharose CL 4B (1.6×60 cm) column eluted at 4°C in 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4. Protein concentrations were determined by the method of Bradford [51] using bovine serum albumin as a standard. Amounts of LDL are expressed as μg of protein. The above density range continues to be widely used (e.g. [52,53]) for the isolation of LDL. Notably, although some size heterogeneity of the isolated lipoproteins is anticipated, this is unlikely to interfere with the present findings. For some experiments, LDL was dialysed against NaCl-free buffer. The mobility and purity of LDL was checked by electrophoresis in 1% agarose gel at pH 8.6 [54]. Polyacrylamide gel electrophoresis performed in the presence of 8% sodium dodecyl sulphate revealed no evidence for degradation of apoB.

2.3. Tryptic digestion of LDL

LDL (0.6 mg protein/ml) was incubated with 2% (w/w) bovine pancreatic trypsin for 16 h at 37°C while an identical sample without trypsin served as a control [55]. Trypsin-treated LDL and control LDL were then adjusted to 1 mM PMSF and fractionated on a column (1.5×23 cm) of Sephadex G-75. The column was equilibrated and eluted with 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4. Fractions of 1 ml were collected and assayed for protein. Those containing LDL were pooled and used in further experiments.

2.4. Oxidation of LDL

LDL was dialysed against 20 mM HEPES, 150 mM NaCl, pH 7.4 at 4°C and its oxidation by cop-

per carried out at 37°C for 24 h in loosely capped glass vials [56]. Concentration of LDL was 2.0 mg protein/ml and [Cu^{2+}] was 20 μM. Oxidation was terminated by the addition of EDTA in excess of the Cu^{2+} concentration. Electrophoresis in 1% agarose gel was performed to verify lipoprotein oxidation [54]. Oxidised LDL was finally dialysed against 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4. Control LDL went through the same procedure, but without exposure to copper.

2.5. Preparation of liposomes

Lipids were dissolved in chloroform and mixed to obtain the desired compositions. They were subsequently dried under a gentle stream of nitrogen and maintained under reduced pressure for at least 2 h to ascertain removal of residual solvent. Unless otherwise indicated, the dry lipid was then hydrated in 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4, to yield a final concentration of 1 mg/ml. In order to obtain large unilamellar vesicles, the hydrated lipids were extruded [57] through 0.1 μm polycarbonate filters (Nuclepore, Pleasanton, CA) with a LiposoFast low pressure homogeniser (Avestin, Ottawa, Canada). The liposomes thus obtained were stored at 4°C and used within 2 days.

2.6. Absorbance measurements

Aggregation of lipid vesicles was monitored by measuring absorbance at 470 nm (A_{470}) using a Shimadzu Graphicord spectrophotometer and cuvettes with an optical path of 1 cm. The cuvette compartment was thermostated with a circulating waterbath either at 15 or 29°C, or as indicated. Prior to the addition of the lipoproteins, the vesicles were allowed to equilibrate for 15 min. Unless otherwise stated, the data shown were corrected for the background absorbance due to LUVs and the lipoproteins used, i.e. the values shown represent increase in A_{470} due to the aggregation of LUVs by LDL. The background absorbances measured for LUVs (typically 75 μM total phospholipid) and LDL (6 μg of protein per ml) were 0.002 and 0.003, respectively. Unless otherwise indicated, the buffer was 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4. In some experiments, PEG (molecular weight of 6000) was included to yield a final concentration of 1% by weight. This [PEG] did not cause any aggregation of LDL or DMPG LUVs as such.

2.7. Lipid mixing assay

Resonance energy transfer between two chromophores (donor and acceptor) observed by steady state fluorescence measurement allows their proximity to be assessed [58]. The radiationless transfer of quanta by dipole–dipole coupling requires the emission spectrum of the donor dye to overlap with the absorption spectrum of the acceptor. Resonance energy transfer assays for monitoring lipid mixing upon vesicle fusion have been devised (e.g. [59]). We utilised, for this purpose, the spectral overlap of excimer emission of the pyrene-labelled lipid analogue, PPDPG and the absorption of the fluorescein-labelled phospholipid derivative, DPPF [60]. Accordingly, if the probes reside within the same membrane, complete disappearance of the excimer band centered at approximately 480 nm results. In contrast, for a mixture of two populations of liposomes, one containing PPDPG and the other DPPF, a strong excimer emission remains. Complete lipid mixing between the two vesicle populations causes quenching of excimer emission (data not shown).

Emission spectra of PPDPG- and/or DPPF-labelled DMPG liposomes were recorded with a Perkin Elmer LS 50B spectrofluorometer connected to a 486, 66 MHz computer. The instrument was operated and the data analysed using the dedicated software from Perkin Elmer. Excitation wavelength was 345 nm, while emission spectra in the range of 360–550 nm were measured. Bandpasses of 2.5 and 3 nm were used for the excitation and emission beams, respectively. Prior to the addition of the indicated lipoproteins, liposomes were allowed to equilibrate for 15 min in a magnetically stirred four-window quartz cuvette in a holder thermostated with a circulating waterbath at 29°C.

2.8. Contents mixing assay

The ANTS/DPX assay described by Ellens et al. [61] for observing mixing of the aqueous contents of LUVs was employed. This method is based on the collisional quenching of ANTS fluorescence by DPX

upon merging of the aqueous cavities during fusion of the two vesicle populations. In brief, DMPG LUVs encapsulating either: (a) 25 mM ANTS (in 40 mM NaCl, 10 mM TES, pH 7.4); (b) 90 mM DPX (in 10 mM TES, pH 7.4); or (c) 12.5 mM ANTS, and 45 mM DPX (in 20 mM NaCl, 10 mM TES, pH 7.4) were prepared. The hydrated lipids were extruded as described above and thereafter centrifuged for 15 min at $10\,000 \times g$ to remove any remaining large, multilamellar liposomes. The ANTS/DPX ratios of 1:1 and 1:0.5 were used for fusion assays. Liposomes with coencapsulated ANTS and DPX were used to set fluorescence to zero. Fluorescence intensities were measured with an SLM 4800S spectrofluorometer using excitation and emission wavelengths of 360 and >530 nm, respectively. Experiments were performed at 29°C in 100 mM NaCl, 5, mM TES, 0.1 mM EDTA, pH 7.4.

3. Results

3.1. Aggregation of DMPG LUVs by LDL

In the course of our studies on the lipid-binding properties of human plasma LDL, we observed this lipoprotein to produce at physiological pH and [NaCl] a rapid aggregation of liquid crystalline DMPG liposomes (Fig. 1A). Measured as a function of time, the increase in absorbance was first preceded by a lag period of up to 15 min, whereafter turbidity increased rapidly, reaching a plateau within approximately 60 min. We did not characterise, at this stage, the kinetics of aggregation in more detail. The lag time seems to vary between 0 to 15 min and it appears to be somewhat dependent on the particular vesicle as well as LDL preparation used. As expected, increasing DMPG concentrations enhanced the rate of aggregation (Fig. 1B). Likewise, using higher concentration of LDL reduced the lag time. Interestingly, aggregation of DMPG by LDL is strongly influenced by the phase state of this lipid and less increase in turbidity was observed when measured at 15°C, i.e. below the main phase transition temperature T_m (=23.5°C) of DMPG (Fig. 1B). Similar observation has been made for the association of apolipoprotein A-I with this phospholipid [62,63].

The dependency on the liquid crystalline state of the liposomes is clearly seen in Fig. 2, which illus-

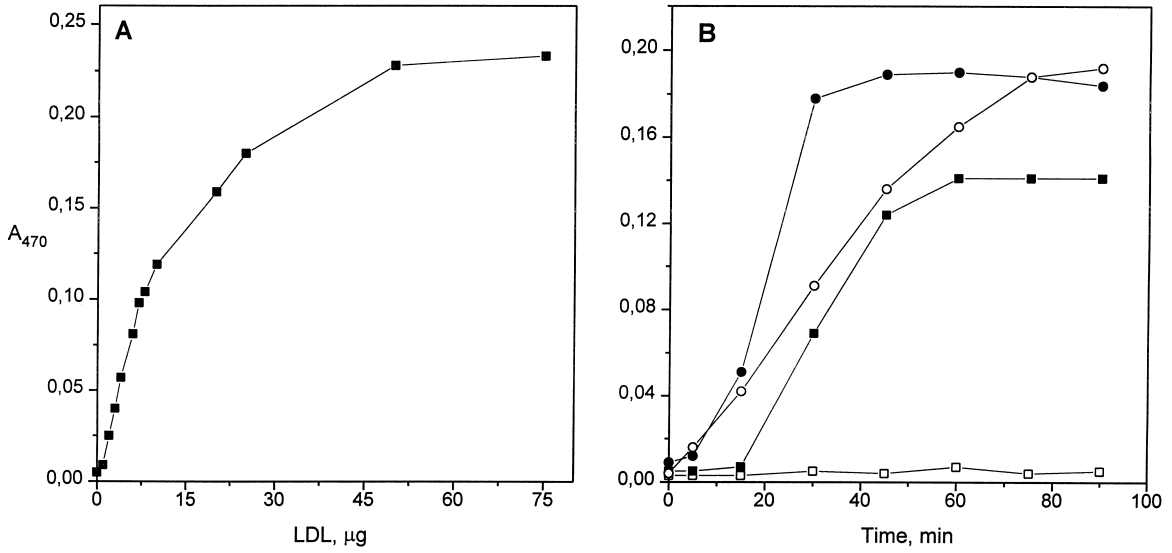

Fig. 1. (A) Aggregation of DMPG liposomes by increasing concentrations of LDL. DMPG liposomes (75 μM) and LDL (up to 75 μg of protein) were incubated in a total volume of 1 ml for 45 min at 29°C (■), whereafter absorbance at 470 nm was measured. Buffer was 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4. (B) The time courses of the aggregation of liquid crystalline and gel state DMPG LUVs. LDL (6 μg/ml) was added to a solution of DMPG LUVs (75 μM) either at 15°C (□) or 29°C (■) while changes in A_{470} were monitored as a function of time. Enhanced aggregation of DMPG LUVs either at higher lipid concentration (225 μM, ●) or using higher concentration of LDL (20 μg/ml, ○) at 29°C was evident. Conditions were otherwise as described for (A).

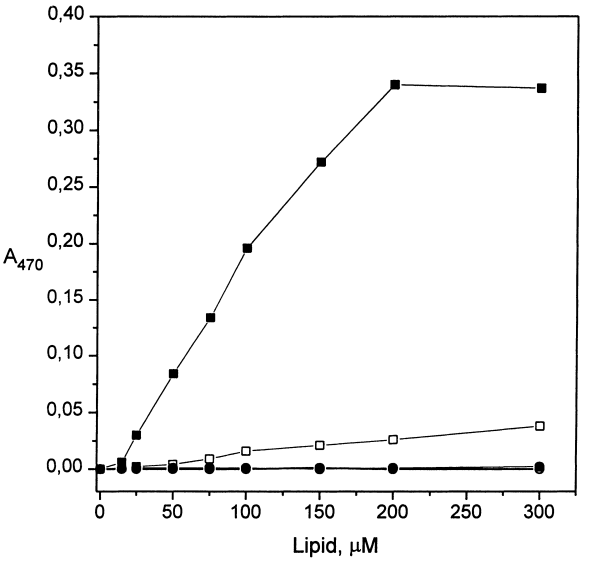

Fig. 2. LDL-induced aggregation of DMPG and DMPC LUVs at varying phospholipid concentrations. LDL (6 μg/ml) was added to DMPG (■,□) and DMPC (○,●) liposomes and A_{470} measured after a 45 min incubation either at 15°C (open symbols) or 29°C (filled symbols). The values for DMPC at both temperatures (○,●) were indistinguishable. The data shown are corrected for the background values of absorbance due to increasing concentrations of LUVs. Buffer was 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4.

trates the aggregation of DMPG LUVs by LDL as a function of lipid concentration, both below as well as above T_m. Although the inclusion of LDL does increase the absorbance of gel state DMPG liposomes, their aggregation is strongly augmented above T_m and the values of A_{470} for liquid crystalline DMPG LUVs were maximally approximately 10-fold higher than those measured below T_m. Due to the well-known electrostatic interaction between LDL and sulphated glycosaminoglycans [18–21], it was readily anticipated that also the aggregation of the acidic DMPG vesicles by this lipoprotein described here would involve coulombic forces. In keeping with this notion, essentially no aggregation due to LDL was evident for LUVs of the zwitterionic DMPC, neither below nor above the main transition temperature of this lipid (Fig. 2). Likewise, increasing the content of DMPC in DMPG LUVs reduced in a progressive manner their aggregation by LDL (see below).

To check the presence of LDL in the aggregates 60 μg of LDL and 840 nmol DMPG were incubated at 29°C for 60 min in a total volume of 1 ml of 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4. The aggregate was sedimented by centrifugation in small plastic vials using a table top centrifuge (Eppendorf) and subsequently analysed for the content of protein. Nearly 70% LDL protein was recovered in the pellet. In comparison, with DMPC no aggregate was formed and LDL protein remained soluble.

3.2. Lack of fusion of LUVs due to LDL

To investigate the possibility that the aggregation of DMPG LUVs by LDL would be accompanied by fusion of the vesicles we employed both lipid as well as contents mixing assays. For the former purpose we prepared liposomes labelled with either PPDPG or DPPF. Subsequently, PPDPG-labelled and DPPF-labelled DMPG vesicles were first mixed, whereafter LDL was added and the emission spectra of pyrene and fluorescein was monitored as a function of time (data not shown). Lipid mixing would be

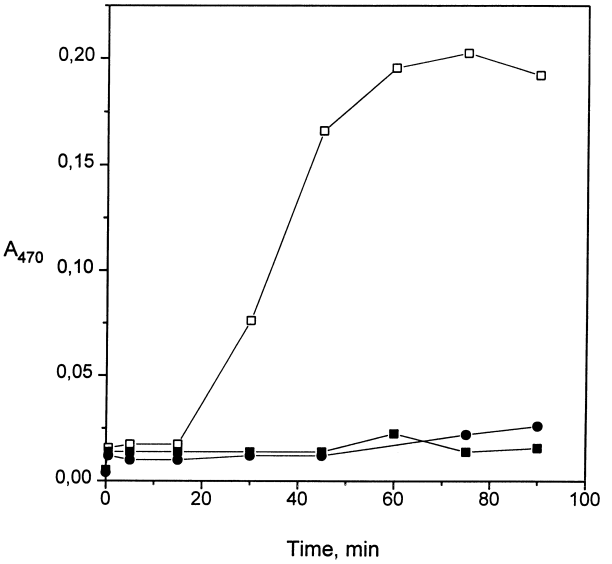

Fig. 3. Lack of aggregation of DMPG liposomes by trypsin-treated and oxidised LDL. DMPG LUVs (75 μM) were incubated with 6 μg/ml of trypsin-treated LDL (■), or 20 μg/ml of oxidised LDL (●) at 29°C and changes in A_{470} were monitored as a function of time. For comparison, experiment with control LDL (6 μg/ml) prepared without trypsin digestion (□) is also shown. Control LDL (20 μg/ml) prepared without $CuSO_4$ gave result similar to that illustrated in Fig. 1B. Medium was 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4.

evident as an effective reduction in the intensity of pyrene excimer emission I_e due to resonance energy transfer to fluorescein [60]. However, intensities of both pyrene monomer and excimer fluorescence were only slightly decreased during a 1 h incubation after the addition of LDL (data not shown). Likewise, during an incubation for 1 h at 29°C the excimer/monomer emission intensity ratio for PPDPG-labelled DMPG LUVs decreased from 0.215 to 0.210 in the absence and to 0.200 in the presence of LDL. Accordingly, although LDL causes the aggregation of DMPG LUVs, lipid mixing does not appear to take place. To this end, the above results are compatible with our measurements revealing lack of aqueous contents mixing [61] of DMPG liposomes upon their aggregation by LDL (data not shown).

3.3. *Role of apoB, comparison of different lipoproteins and the effect of albumin*

Aggregation of DMPG LUVs appears to be a rather specific property of LDL and VLDL, total HDL, and both HDL_2 and HDL_3 subclasses were all essentially ineffective (data not shown). Intact apoB seems to be required and proteolytic digestion of LDL by trypsin [35] fully abolished its ability to aggregate DMPG liposomes (Fig. 3). To this end, also oxidation of LDL by copper [56] completely abrogated the capability of LDL for lipid aggregation (Fig. 3).

While VLDL and HDL do not aggregate DMPG vesicles, it was of interest to study their influence on the aggregation of vesicles by LDL. Notably, the LDL-induced aggregation of DMPG LUVs was effectively inhibited by HDL_3 and to a lesser extent by HDL_2 and VLDL (Fig. 4A). Inhibition was dependent on the amounts of lipoproteins added and at smaller LDL/VLDL and LDL/HDL ratios progressively attenuated aggregation of DMPG LUVs became evident (data not shown). In this context, it is of interest to note that HDL inhibits the aggregation of LDL due to vigorous mixing (vortexing) while the inhibitory mechanism is unclear [34].

We also investigated the effect of bovine serum albumin on the DMPG–LDL interaction (Fig. 4B). When LDL was added to DMPG LUVs in the pres-

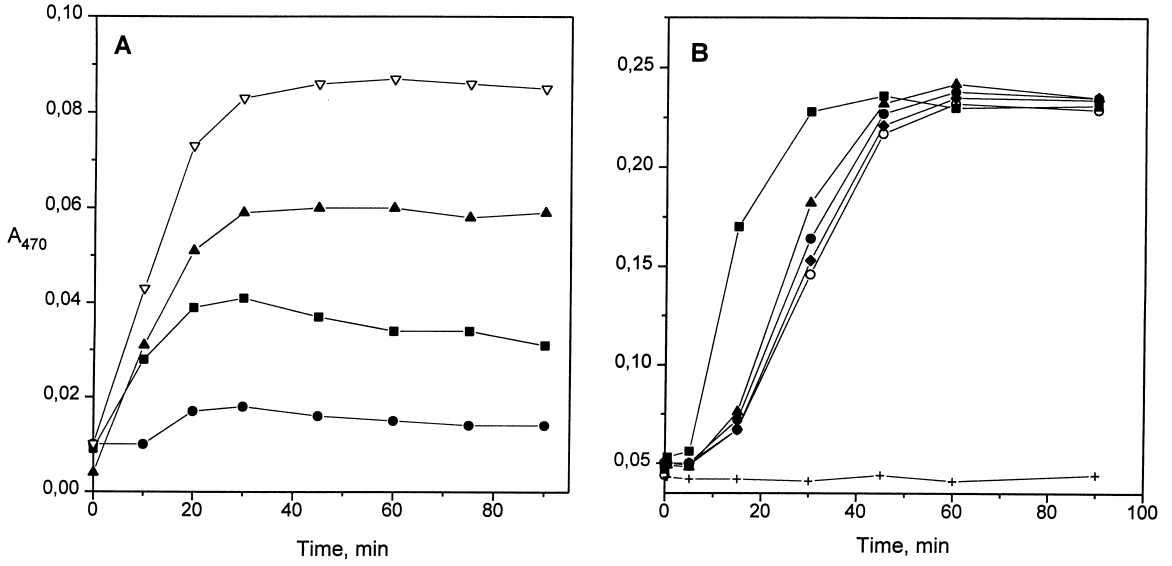

Fig. 4. Inhibition of LDL-induced aggregation of DMPG LUVs by HDL_2, HDL_3, or VLDL, and the effect of BSA. (A) LDL (6 μg) and HDL_2 (■), HDL_3 (●), or VLDL (▲) (6 μg of each lipoprotein) were first mixed in buffer and subsequently (within 10 min) added to DMPG LUVs (75 μM lipid) at 29°C to yield a total volume of one ml, whereafter changes in A_{470} were monitored as a function of time. As a control the aggregation of DMPG LUVs by LDL (6 μg/ml) is depicted (▽). (B) BSA was first added at 29°C to DMPG LUVs (75 μM lipid) to yield the indicated final concentrations. After 15 min LDL (6 μg/ml) was included and A_{470} monitored as a function of time. Concentrations of BSA were 0 (○), 1 (♦), 6 (●), 12 (▲), and 50 μg/ml (■). Data for DMPG LUVs incubated with BSA (12 μg/ml, no LDL) are also shown (+). Buffer in both panels was 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4.

ence of BSA (up to 50 μg/ml, final concentration) aggregation was slightly faster, i.e. the lag time was reduced (in this experiment from approximately 15 to 5 min). Up to highest concentration tested BSA did not cause aggregation of LDL or DMPG as such. The elucidation of the mechanism of this effect of BSA warrants further studies. However, it would be compatible with BSA reducing water activity, thus depleting part of the hydration layer of DMPG and diminishing the energy barrier for the binding of LDL to liposome surface, similarly to the effect of PEG (see below).

3.4. *Effects of ionic strength, pH, and the content of acidic phospholipid in LUVs*

Thermal phase behaviour of DMPG is reasonably well characterised (e.g. [64–66]). Unlike phosphatidylserine or phosphatidic acid the PG headgroup should be less prone to form intermolecular hydrogen bond networks. Furthermore, DMPG does not form non-lamellar phases and its protonation appears to comply with the principles of membrane electrostatics forwarded by Träuble [67]. Accordingly, as the use of PG as a model acidic phospholipid allows for a relatively unambiguous interpretation of the data in terms of changes in lipid protonation [68] we chose this phospholipid for further studies at this stage.

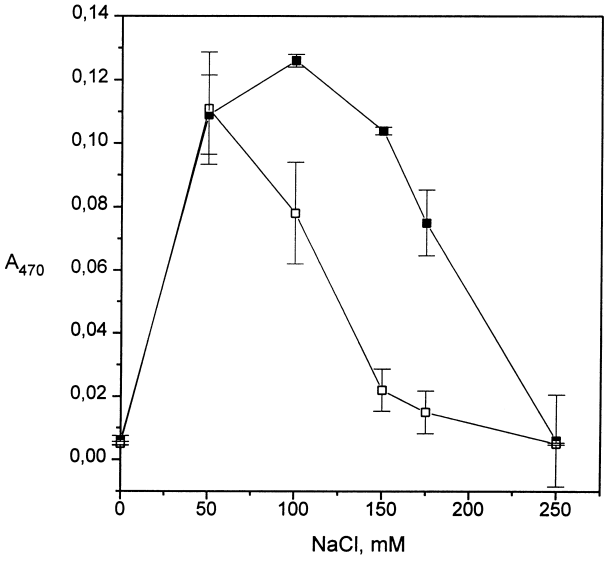

Fig. 5. Effect of [NaCl] on the aggregation of DMPG LUVs by LDL. DMPG liposomes (75 μM) and LDL (6 μg) were incubated for 60 min in a total volume of one ml and in the presence of increasing [NaCl] either at 15°C (□) or 29°C (■), whereafter absorbance at 470 nm was recorded. Buffer was 20 mM HEPES, 0.1 mM EDTA, pH 7.4. Mean ± S.D. for three separate measurements is depicted.

Experiments with LDL treated with trypsin revealed the involvement of apoB in the aggregation process. As described above, aggregation of DMPG liposomes by LDL was evident at physiological [NaCl] and pH. While the net charge of LDL is negative the apoB-100 moiety of LDL contains some positively charged clusters [47,69]. To explore, in more detail, the role of electrostatics in the interaction of LDL with the acidic DMPG, we studied the effect of ionic strength on the aggregation both below and above T_m of the phospholipid, at 15 and 29°C, respectively (Fig. 5). In the absence of salt, no aggregation of DMPG LUVs by LDL was observed, regardless of the phase state of the lipid. Below T_m, maximum aggregation is observed at 25–75 mM NaCl, while above T_m optimum [NaCl] for aggregation is somewhat higher, between 100 and 125 mM NaCl. Below T_m, the lipoprotein induced increase in turbidity was abolished by 150 mM NaCl. In addition, above T_m, the aggregation of liposomes due to LDL did depend on the ionic strength and while considerable aggregation was still seen at physiological [NaCl] concentrations exceeding this were inhibitory. Notably, once DMPG LUVs were aggregated by LDL subsequent addition of salt up to 250 mM failed to reverse the increase in turbidity and essentially no decrease in A_{470} could be observed (data not shown). This irreversibility was evident both below as well as above the phase transition temperature of this phospholipid.

As was already noted above (Fig. 2) DMPC LUVs were not aggregated by LDL. This is also evident in Fig. 6, illustrating progressive reduction in A_{470} upon increase in the content of DMPC in DMPG liposomes. Aggregation of the binary DMPG/DMPC LUVs was sensitive to pH when varied in the range of 5.0 to 7.4 (Fig. 6). More specifically, neat DMPG LUVs were aggregated slightly more efficiently at pH 5 than at pH > 6. Compared to pH 7.4 higher amounts of DMPC in DMPG LUVs were required to prevent the aggregation at acidic pH. Interestingly, acidic pH also prevented the inhibitory effect of POPG on the aggregation of POPG/DMPG LUVs (see below).

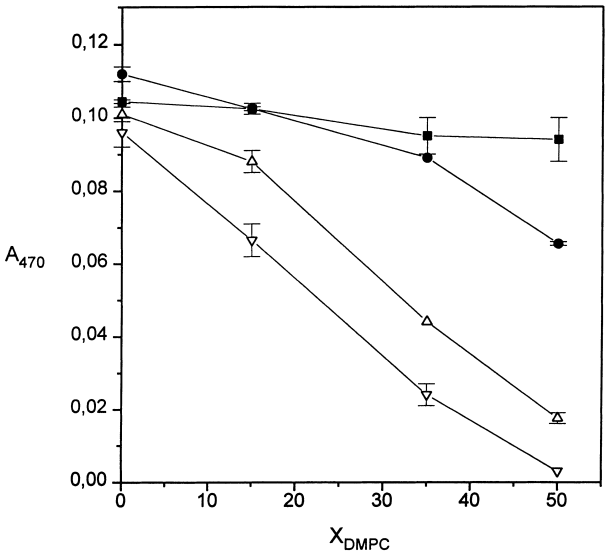

Fig. 6. Effect of pH and the content of DMPC on the aggregation of DMPG LUVs. DMPG liposomes (75 μM) containing from 0 to 50 mol% DMPC were incubated with LDL (6 μg/ml) at pH 5.0 (■), 6.0 (●), 7.0 (△), and pH 7.4 (▽) in a total volume of 1 ml for 60 min at 29°C while monitoring A_{470}. Mean ± S.D. for two separate measurements is shown.

Effects due to changes in bulk pH may have their origin in altered protonation of apoB. Likewise, in addition to simple competition of charges, the above effects of NaCl on the LDL-induced aggregation could also involve changes in the degree of protonation of the acidic phospholipid [67,68]. In order to study the latter possibility, bulk pH was kept at 7.4 and the concentration of DMPG in liposomes maintained constant at 75 μM while the content of DMPC in these LUVs was gradually increased. Accordingly, under these conditions the influence of surface charge density on the aggregation upon varying the content of DMPC can be resolved. These experiments were then performed at varying [NaCl] so as to reveal possible dependency on the protonation state of the acidic phospholipid [67,68]. These experiments were subsequently repeated maintaining the total phospholipid concentration constant (at 75 μM) while again gradually varying the DMPC/DMPG stoichiometry. These two sets of experiments demonstrated that upon increasing the content of DMPG in the bilayers, progressively higher [NaCl] is needed to prevent aggregation by LDL, thus suggesting that an interaction between the protonated lipid phosphate and LDL is involved. Regardless of the DMPG/DMPC molar ratio 175 mM NaCl prevented aggregation (data not shown).

3.5. Comparison of different acidic phospholipids and the effects of PEG and cholesterol

Subsequently, we compared the aggregation of vesicles composed of other saturated acidic phospholipids by LDL (Fig. 7). Both DPPG and DMPS were aggregated by LDL. However, unlike DMPG, they also aggregated in the gel state, i.e. below their T_m values at 41.5 and 39°C, respectively, Interestingly, vesicles composed of unsaturated acidic phospholipids, egg PG, brain PS, and yeast PI were aggregated only weakly or not at all by LDL.

Intriguingly, increasing the content of POPG up to 50 mol% in DMPG LUVs caused progressive inhibition of aggregation prolonging the lag time (Fig. 8A). Analogously to the aggregation of DMPC/DMPG vesicles at acidic pH, also for POPG/DMPG LUVs, rapid aggregation was observed at pH 5.5 (Fig. 8B). Interestingly, at this pH, neat POPG LUVs were also rapidly aggregated due to LDL, although the measured increase in A_{470} was less than for DMPG or the binary vesicles.

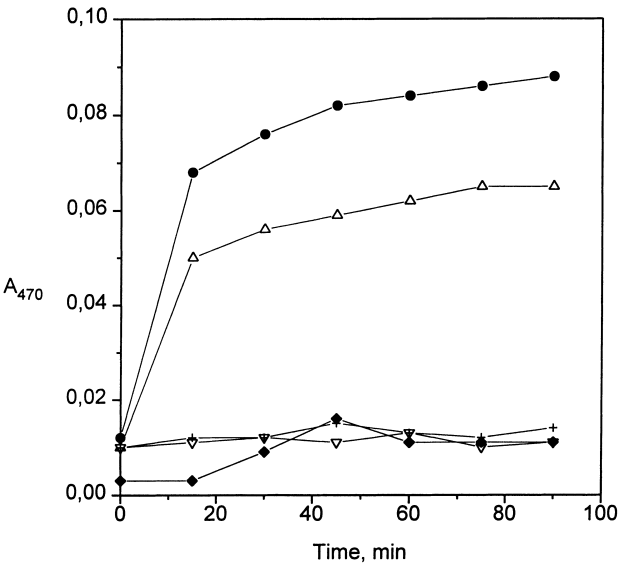

Fig. 7. Aggregation by LDL of LUVs (75 μM) composed of different negatively charged phospholipids, DPPG (●), DMPS (△), yeast PI (+), brain PS (▽), and egg PG (◆). LUVs were mixed with LDL (6 μg/ml) at 29°C, whereafter changes in absorbance were recorded as a function of time. Conditions were otherwise as described in the legend for Fig. 1.

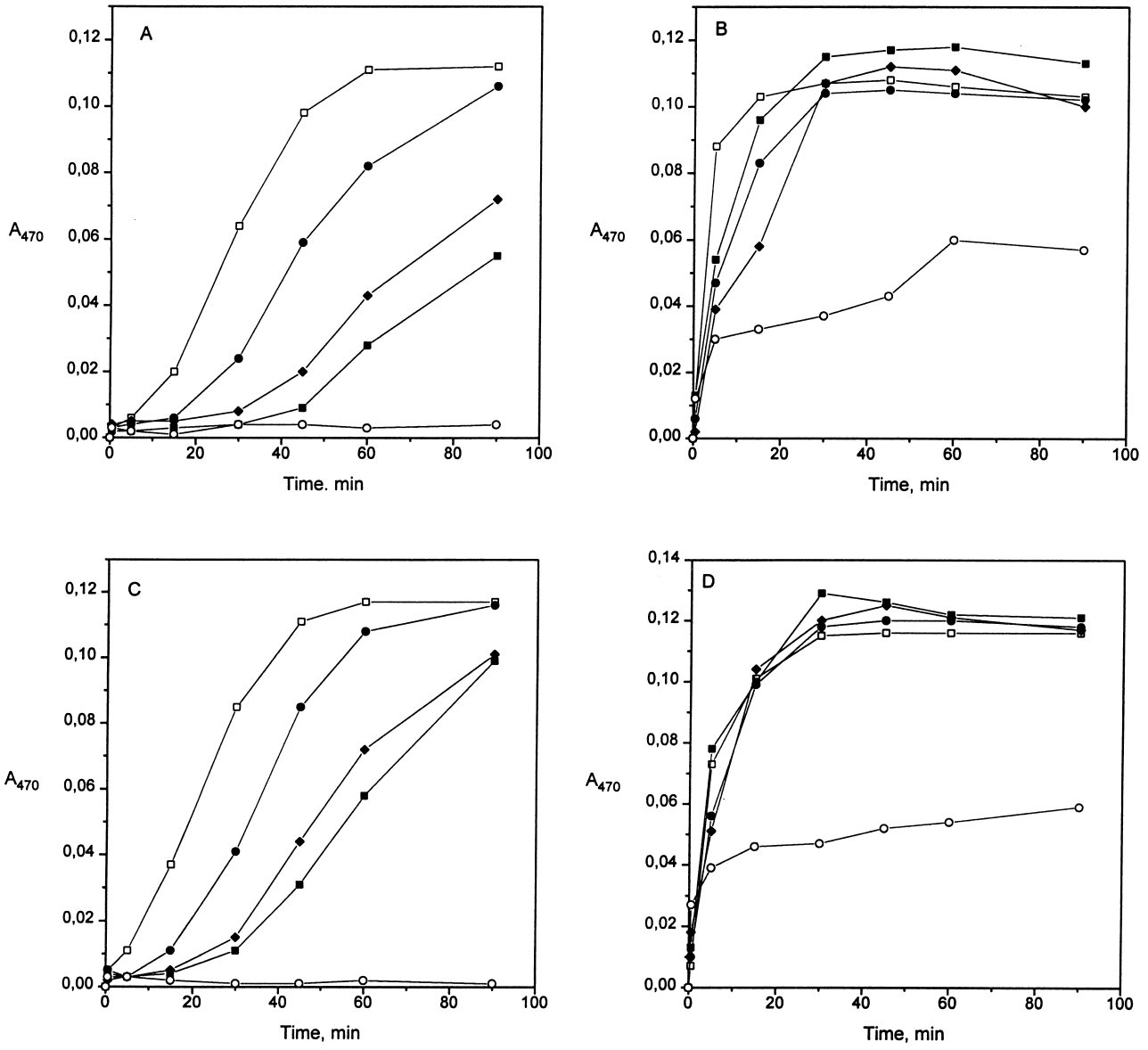

Fig. 8. Dependency of aggregation on the content of POPG in binary vesicles with DMPG, together with the effects of PEG and acidic pH. DMPG liposomes (75 μM total phospholipid) as such (□), neat POPG LUVs (○) or binary vesicles containing 15 (●), 35 (♦), or 50 mol% (■) of POPG were incubated with LDL (6 μg/ml) in the absence or presence of PEG (1%, w/w) at 29°C, whereafter changes in A_{470} were recorded as a function of time. Conditions were as follows: no PEG, pH 7.4 (A), and no PEG, pH 5.5 (B), PEG (1% by weight) at pH 7.4 (C), and 1% of PEG, pH 5.5 (D).

To investigate the role of hydration of the phospholipid vesicle surface as an energy barrier for aggregation we included small concentrations (1% by weight) of the strongly hygroscopic polymer, PEG into the aqueous phase. This [PEG] did not aggregate either DMPG LUVs or LDL separately (data not shown). Yet, it decreased the lag time so that enhanced aggregation of the binary vesicles containing 50 mol% POPG was clearly evident (Fig. 8C). Significant effect due to PEG was observed also at pH 5.5 (Fig. 8D). Finally, similar effects due to PEG were evident also for DMPC/DMPG vesicles (data not shown).

Cholesterol is known to have complex effects on the phase behaviour and physical properties of phospholipid bilayers (e.g. [70–76]). Therefore, it was of

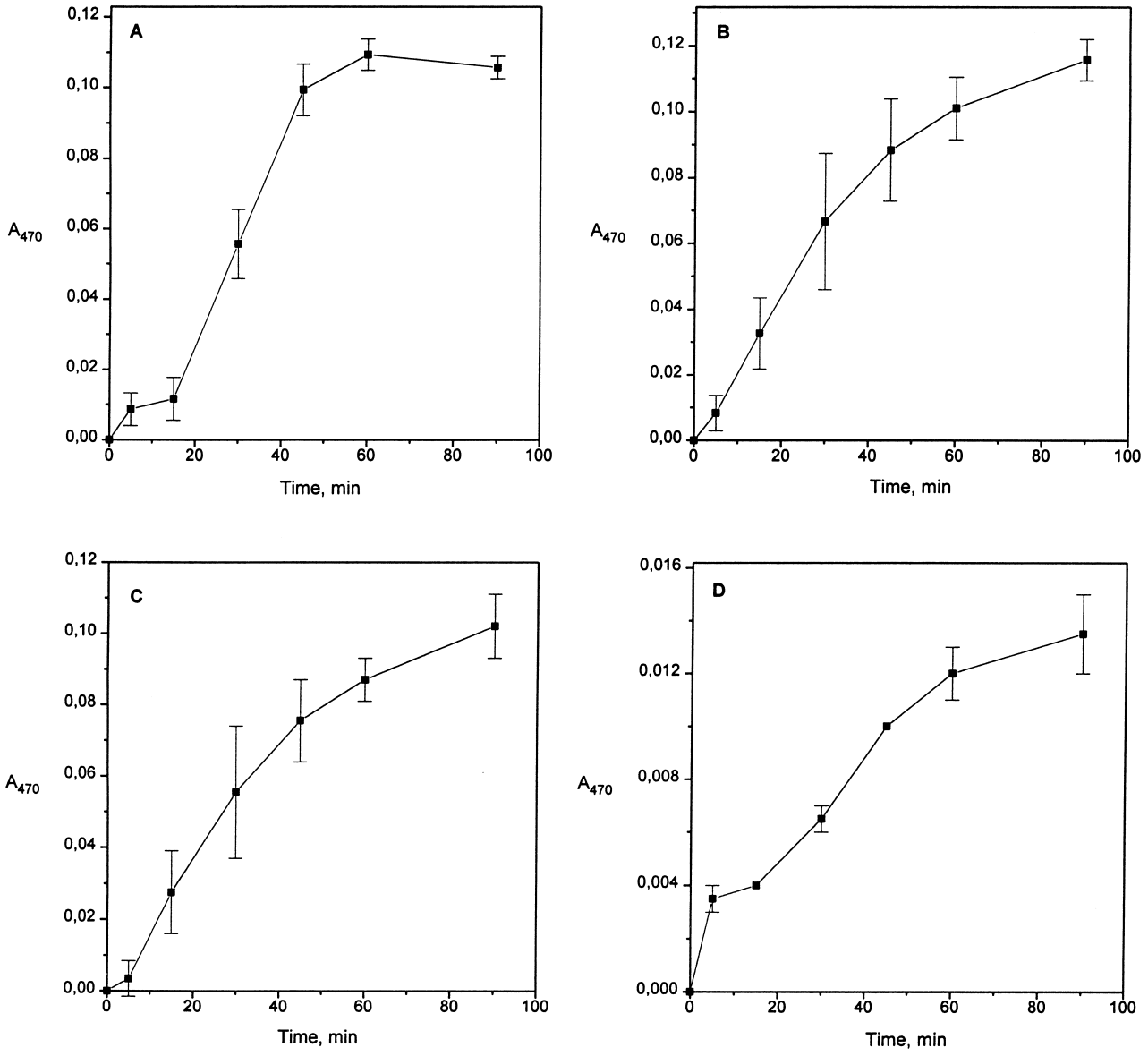

Fig. 9. Aggregation of DMPG/cholesterol LUVs by LDL. DMPG liposomes as such (A) or containing either 25 (B), 35 (C), or 50 mol% (D) of cholesterol were incubated with LDL (6 μg/ml) up to 90 min at 29°C while monitoring A_{470}. In order to show the weak aggregation evident for LUVs containing 50 mol% cholesterol, a different scale had to be used for the y-axis of (D). The concentration of DMPG was maintained constant at 75 μM. Conditions were otherwise as indicated in the legend for Fig. 1. Mean ± S.D. for two separate measurements is shown.

interest to investigate also its influence on the aggregation of DMPG LUVs by LDL (Fig. 9). The apparent rate of aggregation of DMPG vesicles by LDL was enhanced by increasing the cholesterol content X_{CHOL} of the vesicles from 0 to ≈ 0.25 (Fig. 9B) or 0.35 (Fig. 9C) whereas at $X_{CHOL} \approx 0.5$ the time course for aggregation became biphasic (Fig. 9D).

4. Discussion

The present results reveal liposomes composed of saturated acidic phospholipids to be aggregated by human plasma LDL whereas total HDL, HDL_2, HDL_3, and VLDL were ineffective. The binding of LDL to liposomes appears to require intact apoB as tryptic digestion of LDL abolishes its ability to cause

aggregation. The involvement of apoB in LDL in the aggregation of DMPG is compatible also with lack of aggregation by HDL. Notably, lack of aggregation of DMPG by VLDL does not contradict the involvement of apoB in this process as the disposition of apoB is likely to be different in VLDL and LDL. Thus, internalisation of VLDL by fibroblasts by the apoB-100 receptor mediated endocytosis and the subsequent suppression of hydroxymethylglutaroyl CoA reductase requires prior catabolic processing of VLDL to LDL by lipoprotein lipase, as demonstrated in the in vitro study by Catapano et al. [78]. Upon oxidation of LDL, the particle net charge becomes more negative [79,80]. Accordingly, if the sites of apoB responsible for interaction with acidic lipids are modified by oxidation, we may readily expect impaired aggregation.

Preceding aggregation, binding of LDL to the liposome surface must take place. The initial contact of LDL and LUVs is likely to be mediated by an electrostatic attraction between the positively charged residues of LDL (apoB) and deprotonated acidic phospholipids, as indicated by the requirement for the presence of moderate [NaCl] (Fig. 5). Although salt-induced changes in LDL cannot be ruled out, the importance for lipid deprotonation is suggested by the dependency of the [NaCl] required on the lipid phase state. The liposome-associated LDL should then remain capable of further binding with another liposome, thus liganding two vesicles together. Sufficient number of LDL particles per LUVs (see below) should thus result in LDL-mediated cross-links between liposomes and in aggregate formation. Similar process has been described for streptavidin mediated liganding of biotin-lipid containing liposomes into a 3-D maze [81]. Importantly, the LUVs aggregated by LDL do not merge, as evidenced by the lack of aqueous contents mixing. Furthermore, lack of lipid mixing reveals that there is also no hemifusion of the aggregated bilayers. Using a molecular weight of 2.5×10^3 kDa for LDL, taking 1000 Å for the average diameter of the LUVs, and 75 Å^2 as the mean molecular area per DMPG in the liposomes, the stoichiometry of the reactants producing saturating responses in a typical experiment (i.e. 6 μg of apoB per ml) and 75 μM DMPG) is approximately 13 LDLs per one vesicle. The origin of the lag period observed for the aggregation remains uncertain at this stage. One possibility is that a proper number of LDL on the liposome surface is first required to overcome coulombic repulsion between the negatively charged vesicles. Subsequently, aggregate formation can take place. The latter would thus be limited by the collision frequency between the reactants in two processes, first LDLs and LUVs, and then LUVs with bound LDL. This is compatible with the observation that upon increasing the concentration of either LDL or DMPG LUVs the lag time becomes shorter.

It could be argued that the aggregation would actually result from the transfer of DMPG to LDL, subsequently causing aggregation of the lipoprotein. Several lines of evidence point out this mechanism to be unlikely, as follows. First, the transfer of DMPG is rather slow [82]. Second, inclusion of POPG into DMPG should not suppress the transfer of DMPG. However, already at $X_{POPG} = 0.50$ a strong inhibition of LDL-induced aggregation of LUVs is evident. Third, both DPPG and DPPS should transfer extremely slowly through the aqueous phase, particularly when in the gel state. However, vesicles composed of these acidic phospholipids are vigorously aggregated by LDL also when $T < T_m$.

We have recently provided evidence for two acidic phospholipid binding sites in cytochrome *c*, one associating with deprotonated and the other with protonated acidic phospholipids [68,83,84]. Our present results suggest that also LDL associates with both deprotonated as well as protonated DMPG, as follows. The augmented aggregation of acidic liposomes by LDL at more acidic pH could arise from changes in the protonation of acidic phospholipid. However, as varying bulk pH also influences the protonation of apoB, the possible role of DMPG protonation cannot be resolved on the basis of this type of experiments alone. In order to allow for less ambiguous interpretation of the data, we maintained the bulk pH constant at 7.4 and varied the surface concentration of DMPG (X_{DMPG}) by incorporating progressively increasing amounts of DMPC into the LUVs. These experiments were repeated at varying [NaCl] and revealed that an increase in [NaCl] is required to prevent the aggregation of mixed LUVs containing increased surface concentrations of DMPG in DMPC. These data are in perfect keeping with the involvement of an interaction between LDL and protonated DMPG [67,68].

At physiological [NaCl] the aggregation of DMPG is greatly enhanced above the transition temperature T_m of this phospholipid, whereas strongly attenuated aggregation was observed for the gel state LUVs. Accordingly, it was somewhat surprising that LUVs composed of the acidic, unsaturated phospholipids brain PS, egg PG, and yeast PI were not aggregated by LDL. Instead, DMPS and DPPG vesicles were aggregated, thus suggesting that both an acidic headgroup and saturated fatty acid chains are required. The involvement of the latter structural feature in aggregation is intriguing, in particular, as the inclusion of POPG into DMPG LUVs effectively inhibits their aggregation by LDL. Major changes in the surface charge of the binary DMPG/POPG vesicles at $X_{POPG} = 0.5$ are unlikely. Therefore, impaired electrostatic interaction between LDL and LUVs does not explain the effect of POPG and other mechanisms have to be involved. The hydration shell maintained on phospholipid surfaces [85] contributes a barrier for peripheral membrane associating ligands, including proteins [86]. The requirement for [NaCl] for aggregation (Fig. 5) may, in addition to the deprotonating effect, additionally reflect dehydration of the liposome surface [87]. To this end, compared to disaturated phospholipids, experimental evidence indicates an enhanced affinity of unsaturated phospholipids for water [88]. Accordingly, increasing contents of POPG in DMPG LUVs may augment the affinity of the liposome surface for water to an extent preventing the electrostatic association of LDL with the acidic headgroups. Role of the phospholipid hydration layer in retarding the aggregation of vesicles by LDL is further supported by the finding that both albumin and PEG already at low concentrations enhance the aggregation, i.e. faster aggregation becomes evident in their presence. Affinity of liposomes for water is also enhanced at elevated temperatures [88]. To this end, the role for phospholipid hydration layer in preventing the aggregation of liposomes by LDL is further supported by our preliminary observation on the lack of aggregation of DMPG LUVs at higher temperatures (i.e. above 38°C; Lauraeus and Kinnunen, unpublished results).

Cholesterol is an important modulator of the physical properties of membranes [70]. Increasing contents of cholesterol has been verified by a number of different techniques to alter the properties of the bilayer in a non-linear manner (for a brief review see [89]), and a superlattice model describing the lateral distribution of cholesterol in binary membranes has been recently forwarded [89,90]. Unfortunately, contrasting the large number of studies on the properties of cholesterol/PC bilayers (e.g. [72–75,89–94]) significantly less effort has been devoted to elucidate the effects of cholesterol on acidic phopholipids. Yet, a recent study by Borochov et al. [77] shows that the influence of cholesterol on the thermal phase behaviour of DMPG is clearly different from that on DMPC. We have previously reported cholesterol ($X_{CHOL} = 0.20$ or 0.33) to enhance the aggregation of gel state DMPG liposomes by NaCl, and compared to neat DMPG slightly higher salt concentrations were required for the aggregation of binary liposomes containing cholesterol [71]. At $X_{CHOL} \approx 0.25$ and 0.35 somewhat accelerated aggregation of the binary cholesterol/DMPG vesicles by LDL is evident, whereas at $X_{CHOL} \approx 0.50$ the kinetics of turbidity increase induced by LDL becomes more complex. The large scatter of turbidity readings during aggregation evident in the presence of cholesterol is likely to reflect variation in the lag time. This is supported by the smaller variation of the data both in the beginning of the aggregation as well as when the turbidity changes as a function of time have levelled off. Understanding of the physical basis of the observed effects of cholesterol on the aggregation of DMPG LUVs by LDL clearly warrants further efforts. Interestingly, a recent study shows the binding of blood proteins to liposomes to be strongly reduced by the presence of cholesterol [95].

Possible physiological and pathophysiological significance of our results remains speculative at present. Yet, in this context it is worth noticing that cells maintain a rather stringent ATP-dependent asymmetry of their plasma membrane lipids [96]. More specifically, the outer monolayer is devoid of phosphatidylserine which resides instead in the cytoplasmic leaflet [97–99]. Interestingly, uptake of LDL cholesterol by a mechanism not requiring internalisation of the lipoprotein has been described for luteal cells [100]. Non-receptor mediated processes involved in the metabolism of LDL are gaining increasing attention [101]. To this end, although erythrocytes are not capable of receptor mediated endocytosis, binding of LDL to these cells has been

demonstrated. The binding of LDL to erythrocytes did not depend on the membrane proteins of this cell and instead an enhanced association became evident after the treatment of these cells with proteases [38]. It is tempting to speculate that damage and ultimately necrosis of cells in the arterial wall either due to hypoxia or hypertension may result in loss of membrane phospholipid asymmetry, which could further lead to the binding of LDL to PS exposed in the outer monolayer. Cellular debris is evident upon histological examination of atheroma [102]. The present study further demonstrates the association of LDL to acidic phospholipids to be enhanced by the protonation of the latter. Accordingly, binding of LDL to acidic phospholipids should be further augmented at acidic pH, suggested to prevail at sites of ischaemia and cell injury [103–105]. Formation of domains [106] enriched in acidic phospholipids would further enhance this interaction.

Due to the increased risk for atherosclerosis associated with oxidation of LDL, the lack of aggregation of liposomes composed of acidic phospholipids reported here by this modified LDL might at first appear to be paradoxical. The acidic pH prevailing inside of lysosomes, together with the rather high content of acidic phospholipids in these organelles [107,108], should favour direct association of the internalised LDL with lysosomal membranes. Accordingly, it is possible, in principle, that this interaction could contribute to the metabolism of LDL within lysosomes. Our recent experiments indicate that LDL may actually fuse with liposomes containing acidic phospholipids, resulting in the transfer of lipids from LDL into the liposomal bilayer (Lauraeus and Kinnunen, unpublished data). If the interaction of LDL with acidic phospholipids is involved in its intralysosomal catabolism, impaired processing of oxidised LDL and elevated risk for atherosclerosis would be anticipated. Studies exploring these possibilities are now in progress in our laboratory.

Recent evidence suggests the involvement of the respiratory pathogen *Chlamydia pneumoniae* strain TWAR, an obligatory intracellular Gram-negative bacteria, in the development of coronary heart disease as well as asymptomatic atherosclerosis (e.g. [109–112]). However, the mechanistic link connecting atherosclerosis and *C. pneumoniae* infection has remained elusive. To this end, the major lipids of Gram-negative and -positive bacteria are phosphatidylethanolamine and PG with a significant portion of lipids being saturated [113]. The present data suggest the intriguing possibility that co-aggregation of LDL and *C. pneumoniae* could contribute to the development of atheroma. This notion is of particular interest as negatively charged phospholipids have been shown to form a complex with LDL [48]. Furthermore, this complex is phagocytosed at a higher rate by macrophages. Studies on the aggregation of *C. pneumoniae* by LDL are currently in progress in our laboratory.

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