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Activity of mammalian secreted phospholipase A_2 from inflammatory peritoneal fluid towards PEG-liposomes. Early indications☆

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Abstract

Due to an increase in the activity of phospholipase A_2 (PLA_2) in various inflammatory diseases, this enzyme may play a key role in the degradation of liposomes and the subsequent release of drug when PEG-liposomes passively target inflammatory tissue. The activity of mammalian secreted phospholipase A_2 ($sPLA_2$) in casein stimulated peritoneal fluid was tested toward liposomes of different compositions. Early results indicate only a slight degradation of conventional dipalmitoylphosphatidylcholine (DPPC) liposomes as well as DPPC liposomes incorporated with different concentrations of PEG_{2000}. However, the DPPC degradation increased to 7% when inclusion of 30 mol% phosphatidylethanolamine (PE) in the lipid bilayer. The increase in degradation may be due to an improvement of the substrate — as it is well known, that PE is a better substrate for the mammalian $sPLA_2$ than PC. Incorporation of PE into the bilayer may increase the binding properties of the bilayer resulting in improved conditions for the enzymatic attack by $sPLA_2$. In addition, inhibitory zones of *Staphylococcus aureus* in an agar diffusion test showed that PLA_2 from *Crotalus atrox* venom was able to catalyze the release of gentamicin from PEG-liposomes. In conclusion, this study suggest that degradation of the lipid bilayer of PEG-liposomes by PLA_2 result in release of incapsulated drug, e.g. gentamicin and inclusion of PE in the liposomal bilayer, may enhance the activity of the mammalian $sPLA_2$ toward liposomes composed of DPPC.

Keywords: Drug delivery; PEG-liposomes; Phspholipase A_2; Lipopolymer; Liposomes; Degradation

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1. Introduction

Degradation of liposomes by the specific phospholipases in the bloodstream may be minimal due to the low amount of these enzymes (Senior, 1987). However, due to an enhanced level of mammalian secreted phospholipase A_2 ($sPLA_2$) in different pathological tissues (Jain and Berg, 1989; Gelb et al., 1994; Fourcade et al., 1995; Jamal et al., 1998), e.g. inflammation and cancer, this enzyme may have a marked effect on liposomal degradation at these target sites. Due to the steric barrier the long circulating PEG-liposomes tend to escape from the clearance mechanisms in the bloodstream and accumulate in the more permeable pathological tissues.

In contrast to what was generally expected, it has recently been found that the degree of lipid hydrolysis catalyzed by PLA_2 from *Naja naja* venom increased with increasing amounts of lipopolymers in the liposomal bilayer (Vermehren et al., 1998). Hence, PEG was found to activate the PLA_2-catalyzed liposomal hydrolysis in vitro.

Thus, it may be hypothesized that the accumulation of the long circulating PEG-liposomes in inflammatory tissue may result in destabilization of the liposomes with the subsequent specific release of incapsulated drug at the target organ due to a local high concentration of PLA_2.

$SPLA_2$'s are a family of low molecular weight, calcium dependent, extracellular, interfacial active enzymes (Rice, 1998). They hydrolyse the sn-2 acyl ester bond of 1.2 diacyl-sn-3 phosphoglycerides, liberating equimolar amounts of 1-acylglycerophosphatide and free fatty acid. Type II PLA_2 has been isolated from peritoneal exudate, rheumatoid arthritic synovial fluid, platelets, spleen, intestine, ascites and placenta, and have been reported to be released from many cell types (Rice, 1998). Type II PLA_2 enzymes does not exhibit a sn-2 fatty acid substrate preference — however are selective for aminoglycerophospholipids (Fourcade et al., 1995; Rice, 1998).

Once released, $sPLA_2$ may affect cell function via interaction of a cell-surface receptor that activates intracellular second messengers, and via hydrolysis of cell membrane phospholipids liberating metabolites, which may lead to the formation of phospholipid-derived mediators. Besides this, the activity of $sPLA_2$ may help to prevent the accumulation of aminophospholipids on the cell surface hereby preventing fusion of cell membranes.

Under normal conditions the outer layer of the cell membrane is primarily composed of the neutral phosphatidylcholines (PC). Thus, the cell represents an inert surface to the environment. The negatively charged aminophospholipids are located in the inner leaflet of the cell membrane due to the maintenance of the asymmetric distribution of phospholipids across the cell membrane. Appearance of the aminophospholipids in the outer layer may lead to increase in PLA_2 activity in order to maintain an inert surface of the cell membrane (Fourcade et al., 1995). Hence, it may be possible to optimize the liposome composition in order to obtain an improved substrate for the PLA_2 attack by incorporating phosphatidylethanolamine (PE) into the PC bilayer.

Thus, the aim of this work is to obtain a source of $sPLA_2$ in an inflammatory exudate in order to gain further insight into the mechanisms controlling destabilization of liposomes and drug release at the target site, e.g. inflammatory tissue. The aim of this study is to include experiments illustrating the sufficiency of the $sPLA_2$ to degrade liposomes as well as the effect of PEG on the enzymatic activity.

2. Method

Casein stimulated peritoneal exudate was isolated from rats 8 h after interperitoneal (i.p.) injection of 30 ml 1% sodium caseinate. The peritoneal cavity was washed with 25 ml NaCl including heparin (2 U/ml). To obtain cell-free peritoneal fluid the cells were spun down at $160 \times g$ (1200 rpm) for 7 min. The supernatant was recentrifugated at $1700 \times g$ (4000 rpm) for 7 min.

Unilamellar liposomes with a size of 100 nm were produced as earlier described (Vermehren et al., 1998; Schiffelers et al., 1999). Liposomes composed of dipalmitoylphosphatidylcholine (DPPC) including PE-PEG_{2000} were used for the $sPLA_2$ exudate assay. Assay conditions for the $sPLA_2$ catalyzed phospholipid hydrolysis included 50

nmol phospholipid substrate (liposomes/ultrasonicated phospholipid) including 400 dpm [3H]DPPC per nmol phospholipid, 50 mM Tris–HCl buffer (pH 7.0), 4mM $CaCl_2$, 5 μl peritoneal exudate/fluid in a total incubation volume of 250 μl. The liposomes were incubated for 1 h at 37°C. The reaction was stopped by 0.5 ml chloroform/methanol/acetic acid (2:4:1, v/v/v). The lipids were extracted by 0.5 ml chloroform/methanol (2:1, v/v) and the potential phospholipase mediated metabolites were separated by thin layer chromatography (TLC) using a solvent system of chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v). The radioactivity of the individual scraped lipid spots were measured by the liquid scintillation counting.

For the agar diffusion test a *Staphylococcus aureus* suspension was spread over the agar in the petri-dish. Nine wells were punched in the agar. Five were filled with the standard concentrations of 0.5/1/2/4 and 8 ug/ml gentamicin. The wells indicated with '+C' contain liposomes with cholesterol (with the composition partially hydrogenated egg PC:cholesterol:distearoylPE-PEG_{2000} (1.85:1:0.15 mol:mol)). Liposomes without cholesterol with the composition egg PC:distearoylPE-PEG_{2000} (2.85:0.15 mol:mol) were present in wells marked '−C'. All liposome suspensions were in Ca^{2+}-containing buffer with 1% BSA. Addition of PLA_2 to liposomes was indicated by '+P'. For each lipid composition the incubation was performed in a total volume of 25 ml at 37°C, and included 15 μmol lipid, 1125 ug liposome-encapsulated gentamicin and, when indicated, PLA_2 from *Crotalus atrox* venom (1 U/ml). Samples were taken at 1, 4, 8 and 24 h. The agar dish was incubated overnight at 37°C.

3. Results and discussion

In the agar diffusion test, increasing inhibitory zones (black circles) surrounding the wells were associated with increasing concentrations of gen-

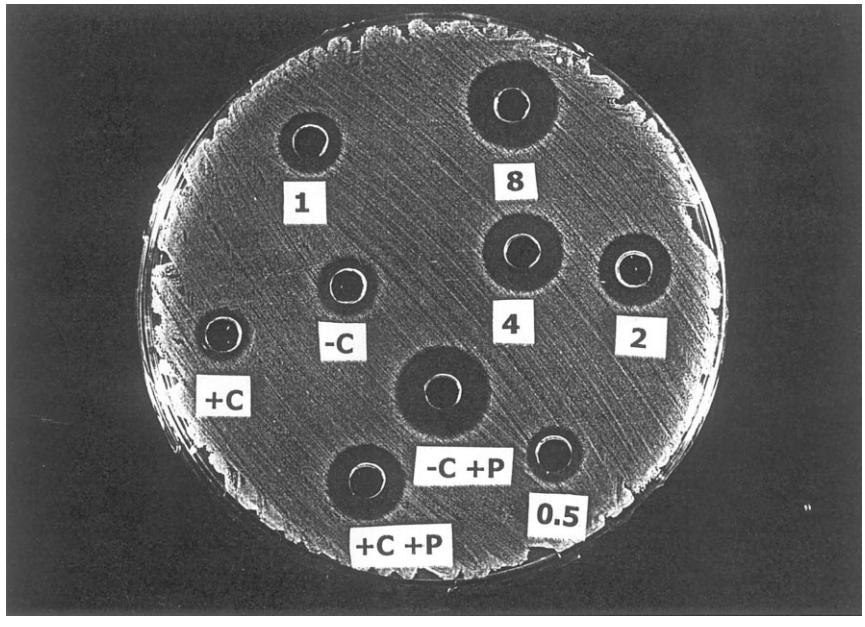

Fig. 1. Agar diffusion test showing inhibitory zones of *S. aureus* caused by gentamicin release, 0.5, 1, 2, 4, 8, punched wells in the agar filled with gentamicin standards (0.5, 1, 2, 4, 8 μg/ml). +C, wells filled with liposomes of the composition partially hydrogenated egg phosphatidylcholine:cholesterol:distearoylphosphatidylethanolamine (1.85:1:0.15 mol:mol). −C, wells filled with liposomes of the composition egg phosphatidylcholine:distearoylphosphatidylethanolamine (2.85:0.15 mol:mol). +P, incubation of liposomes with phospholipase A_2 (1 U/ml).

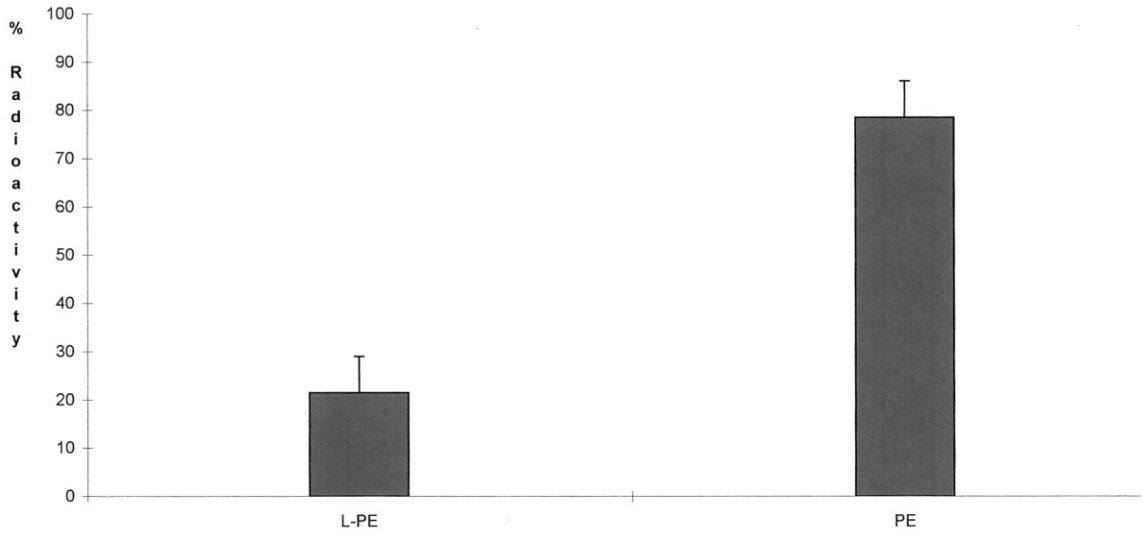

Fig. 2. Activity of secreted phospholipase A_2 in casein stimulated cell free peritoneal fluid toward PE. Production of lysophosphatidylethanolamine (L-PE). Values are mean ± S.E.M. ($n = 2$).

tamicin standards (Fig. 1). A small percentage of the liposome associated gentamicin is located outside the liposome resulting in a small inhibitory zone both for liposomes containing cholesterol and without cholesterol (wells marked + C, − C). Thus, the liposomes showed hardly any leakage. However, after the addition of 1 U/ml PLA_2 from *C. atrox* venom (+ C + P, − C + P) the inhibitory zones were increased manyfold indicating a PLA_2 mediated gentamicin release. In this test, samples taken at 1, 4, 8 and 24 h all yielded the same results.

The casein stimulated peritoneal fluid was assayed for $sPLA_2$ activity by using ultrasonicated, radiolabelled PE as substrate. The formation of radiolabelled lysoPE indicated $sPLA_2$ activity. During 1 h 21.5 ± 7.5% (S.D.) of the radioactivity corresponded to the degradation product (Fig. 2). The degradation rate is in accordance with the rate of degradation of PE by $sPLA_2$ in casein stimulated peritoneal fluid observed by Chang et al. (1986). In this study, Chang et al. (1986) showed that the activity of $sPLA_2$ increased as a function of time and paralleled the increase in the number of polymorphonuclear leukocytes in the peritoneal cavity. The highest concentration was 40 × elevated compared with the activity of $sPLA_2$ in peritoneal fluid obtained from untreated rats. This concentration appeared 8 h after the casein injection (Chang et al., 1986). However, only a slight degradation of DPPC-liposomes was observed in this study, when incubated with the full peritoneal exudate containing polymorphonuclear leukocytes (Fig. 3). Incorporation of 5 mol% PE-PEG_{2000} into the liposomal bilayer did not facilitate the liposomal degradation (Figs. 3 and 4) and the degradation rate did not seem to be improved by incorporation of 2.5 or 7 mol% PE-PEG_{2000} (not shown). Similar, incubation of DPPC-liposomes and PEG-liposomes with homogenized exudate and the cell free peritoneal fluid did not show a degradation different from the control (NaCl stimulated peritoneal fluid). However, the $sPLA_2$ activity toward DPPC liposomes include 30 mol% PE was increased compared with the pure DPPC liposomes. The production of lyso PC from these liposomes increased to 6.5 ± 0.4% (S.D.) of the total radioactivity.

The results suggest that unilamellar 100 nm DPPC liposomes are a poor substrate for the $sPLA_2$. It is well known, that the $sPLA_2$ show a

marked preference for PE with only low activity being expressed using a PC substrate (Vadas et al., 1993; Kinkaid and Wilton, 1994). The different $sPLA_2$'s display different interfacial binding properties and it has been shown, that the human group II $sPLA_2$ bind only weakly or not at all to vesicles of PC (Bezzine et al., 2000). Of all the $sPLA_2$'s analyzed, cobra venom $sPLA_2$ has been shown to display the highest affinity of the zwitterionic PC vesicles (Vadas et al., 1993). The observed increase in the $sPLA_2$ mediated degradation of DPPC liposomes including PE may be caused by an increase in the interfacial binding to the vesicles. It has shown earlier that inclusion of anionic phospholipids such as cholesterol sulphate in PC vesicles caused enhanced interfacial binding and hydrolysis of PC by the human enzyme (Kinkaid and Wilton, 1998).

It is believed that this enzyme may act as an acute phase protein that is released in trauma (Kinkaid et al., 1998). The role of the enzyme may be to assist in the degradation of infectious organisms and damaged cells and a characteristic of such an extracellular enzyme should be the inability to degrade the plasma membrane of normal cells. The anionic phospholipids and PE are located in the inner leaflet of the cell membrane — however, by scrambling PE might translocate to the outer leaflet. Thus, mimicking a scrambled cell membrane by incorporation of anionic phospholipids and PE into the PC membrane of the liposomes may improve the liposomes as a substrate for the $sPLA_2$ in inflammatory tissue and it is conceivable that a PLA_2-catalyzed degradation of the liposomes in the target tissue may cause release of drug at the pathological site.

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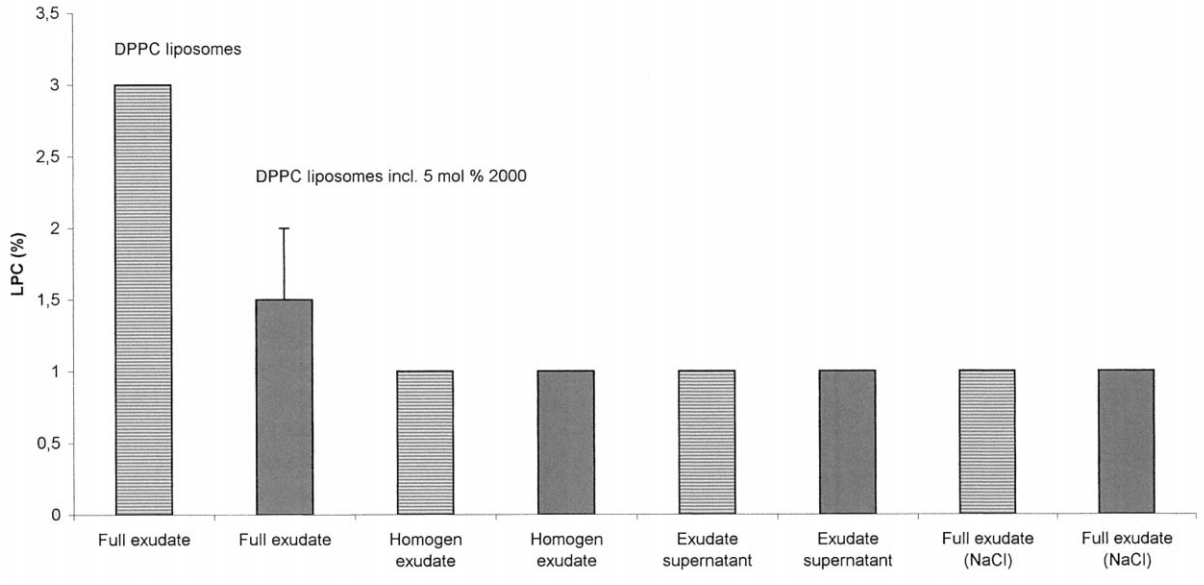

Fig. 3. Activity of secreted phospholipase A_2 in peritoneal exudate/fluid stimulated with casein or NaCl toward DPPC liposomes and PEG-liposomes. Production of lysophosphatidylcholine (LPC). Values are mean $\pm$ S.E.M. ($n = 2$).

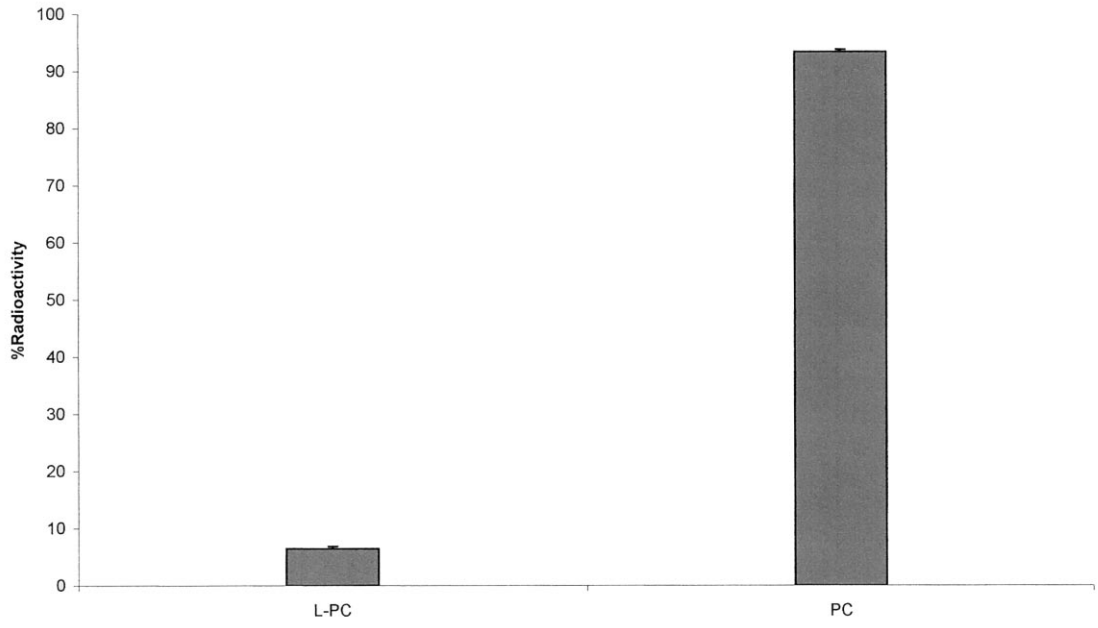

Fig. 4. Activity of secreted phospholipase A_2 toward DPPC-liposomes including 30 mol% PE in casein stimulated peritoneal exudate. Production of LPC. Values are mean ± S.E.M. ($n = 2$).

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