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Antibodies and recombinant standards for the Lp-PLA2 fluoroimmunoassay

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Introduction

Lp-PLA2 (which is also known as platelet-activating factor acetyl hydrolase) is a Ca^{2+}-independent phospholipase that is produced by monocytes and macrophages, and which circulates in the bloodstream in the form of a complex with lipoprotein particles (**1**). Lipoprotein-associated phospholipase A2 plays a crucial role in the metabolism of pro-inflammatory phospholipids as well as in the generation of pro-atherogenic metabolites, such as lysophosphatidylcholine and oxidized free fatty acids. Lp-PLA2 mostly binds to LDL while the remaining part is bound to HDL and the distribution of Lp-PLA2 over lipoprotein classes correlates with the pathophysiological state. The association of Lp-PLA2 with lipoproteins is dependent on its glycosylation at specific residues (**1**).

Lp-PLA2 levels have been shown to predict adverse cardiac-related events in patients with stable coronary artery disease and in a healthy adult population. Recent guidelines from the European Society of Cardiology, the American College of Cardiology, the American Heart Association and the American Society of Endocrinology included the Lp-PLA2 levels measurement among the biomarkers that are useful for the risk stratification of asymptomatic adult patients. Mass concentration of the Lp-PLA2 in human serum could be measured by means of immunoassays.

The aims of this study were:

- To develop antibodies capable of detecting Lp-PLA2 with high sensitivity
- To develop mAb pairs that recognize native Lp-PLA2 as part of lipoprotein complexes
- To develop recombinant antigen with immunochemical properties that are similar to those of native Lp-PLA2

Methods

Recombinant human Lp-PLA2 was produced in two expression systems: mammalian cell line and insect cell line. The recombinant proteins were purified from conditioned media by several chromatographic steps. Purified proteins were then further utilized as immunogens in murine monoclonal antibody development by utilizing hybridoma technology. The selection process for antibody-producing hybridomas included the ELISA method with the human cell line derived Lp-PLA2 preadsorbed onto the plate surface.

Those antibodies that demonstrated good sensitivity were taken for further MAb combination testing in the sandwich ELISA.

Having selected the best MAb pairs in vitro with recombinant human Lp-PLA2, we studied native human Lp-PLA2 utilizing the MAb combinations selected. Admission serum samples from patients with acute myocardial infarction (N=13) and serum samples from apparently healthy adult volunteers (N=13) were used in the study.

The distribution of recombinant and native Lp-PLA2 immunoreactivity over elution profiles was studied by utilizing size-exclusion chromatography. 150 Ql of serum sample or recombinant human Lp-PLA2 solution was applied onto the Superose 6 column that was connected to the AKTA pure chromatography system. 400 Ql fractions were collected and immunoreactivity in fractions was determined by utilizing the MAb combinations indicated. To study the recovery of recombinant Lp-PLA2 in human serum, 3 Qg of recombinant Lp-PLA2 was added to 150 Ql of normal human serum and incubated for 15 minutes at room temperature. Serum spiked with recombinant Lp-PLA2 was applied onto the Superose 6 column in the same way as normal human serum.

For the dilutional linearity experiment, pooled serum samples from healthy volunteers were diluted stepwise along with recombinant Lp-PLA2 calibrator. The immunoreactivity profile in the dilution series was determined by utilizing the MAb combination indicated.

Results and discussion

Recombinant Lp-PLA2 has been successfully expressed in both mammalian and insect cell lines. Based on the assumption that the glycosylation status of human cell line-developed protein is closest to that of native Lp-PLA2 in the bloodstream, we have put together a selection of mammalian cell line produced recombinant Lp-PLA2 as an immunogen and immunoassay calibrator. By employing two chromatographic methods, we isolated recombinant Lp-PLA2 from conditioned media with >75 % purity.

Using purified recombinant Lp-PLA2, we developed a panel of murine monoclonal antibodies that are capable of reacting with recombinant Lp-PLA2 preadsorbed onto a plate surface. For further testing, 28 antibodies were combined in pairs and tested in sandwich ELISA. Seven MAbs constituting five MAb pairs were selected based on their performance in sandwich ELISA. Interestingly, the selected MAb pairs recognized both mammalian cell line-derived and insect cell line-derived recombinant Lp-PLA2 proteins in a similar manner. Two MAb combinations - PL42-PL46 and PL26-PL4 - demonstrated sensitivity of 0.2 and 0.5 ng/ml respectively and a good linearity range (1-1000 ng/ml for the MAb combination PL42-PL46) (see Figure 1).

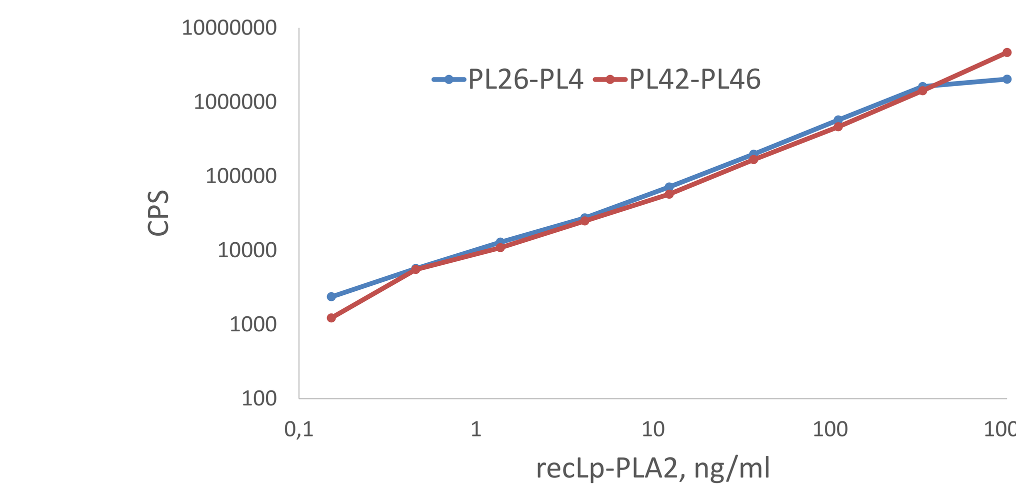

FIGURE 1. Recombinant Lp-PLA2 titration curves in a sandwich ELISA using PL42-PL46 and PL26-PL4 MAb combinations. The capture antibody PL42 or PL26 was coated onto the wells of a Costar EIA/RIA plate. The plate was blocked with a buffer containing 1% casein and 0.05% Tween 20 at room temperature for 15 minutes. Recombinant human Lp-PLA2 and the detection MAb PL46 or PL4 labelled with europium chelate were diluted in an assay buffer and incubated in coated plate wells for 2.5 hours at 37°C.

To study the interaction of selected MAbs with native human Lp-PLA2, we determined the distribution of immunoreactivity measured with the MAb combination PL42-PL46 over an elution profile of size-exclusion chromatography when normal human serum was applied onto the matrix. There are three distinct peaks of immunoreactivity in fractions after normal human serum gel-filtration (see Figure 2 A). If we spike the normal human serum with recombinant Lp-PLA2, immunoreactivity in fractions was increased and there was no shift in peak elution volumes (see Figure 2 B), which indicates that recombinant phospholipase was associated with the same lipoprotein particles as native Lp-PLA2.

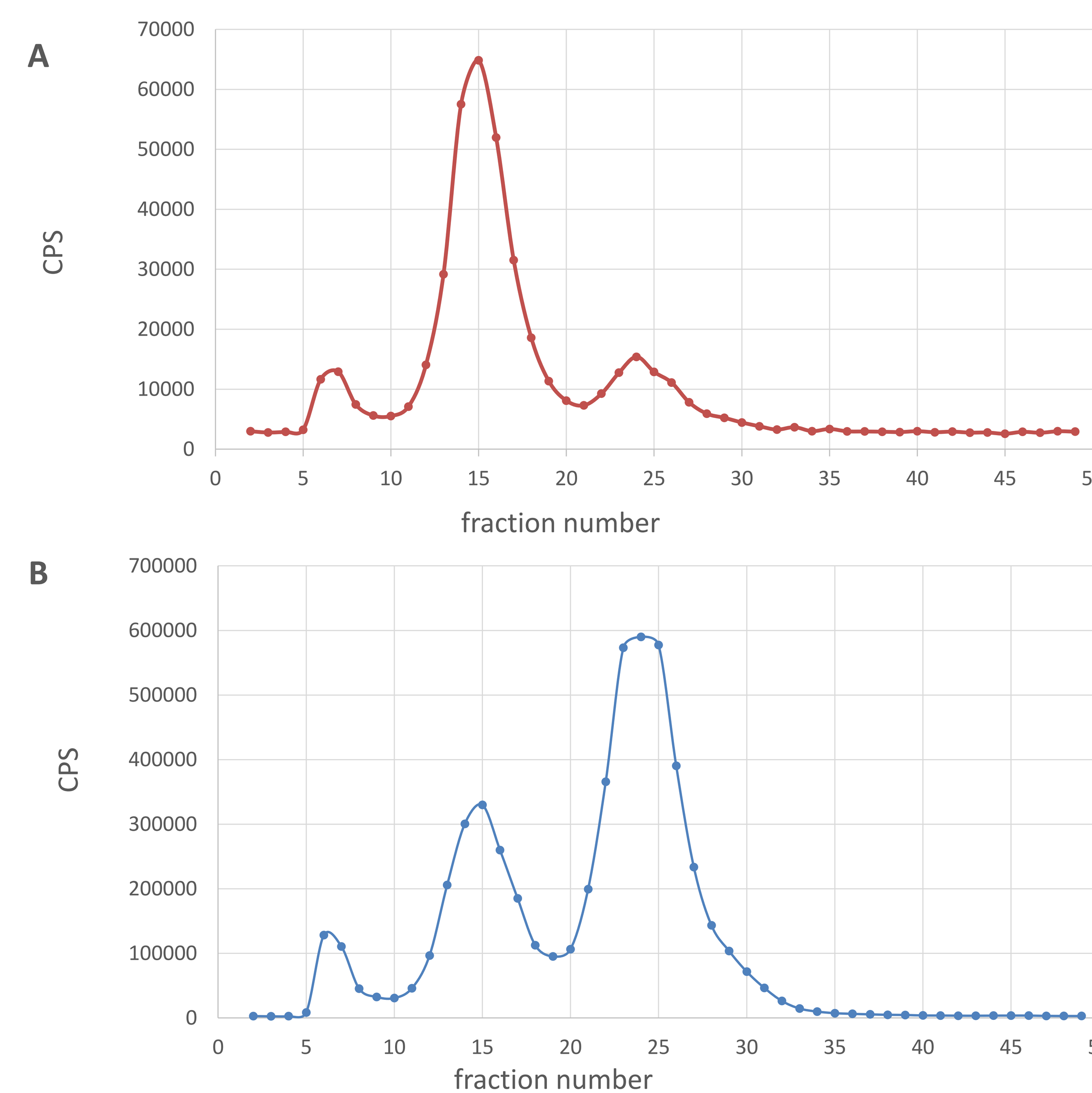

FIGURE 2. Distribution of Lp-PLA2 immunoreactivity over an elution profile during gel-filtration. 150 Ql of serum (A) or 150 Ql of serum spiked with recLp-PLA2 (B) was applied onto the Superose 6 gel-filtration column. Immunoreactivity in fractions was determined in a sandwich ELISA by utilizing the assay PL42-PL46.

In another set of experiments we titrated normal human serum alongside recombinant Lp-PLA2 in order to check curves for parallelism. Both specimens were titrated in a similar manner which indicated that recombinant Lp-PLA2 is close to its native counterpart in immunoreactivity (see Figure 3).

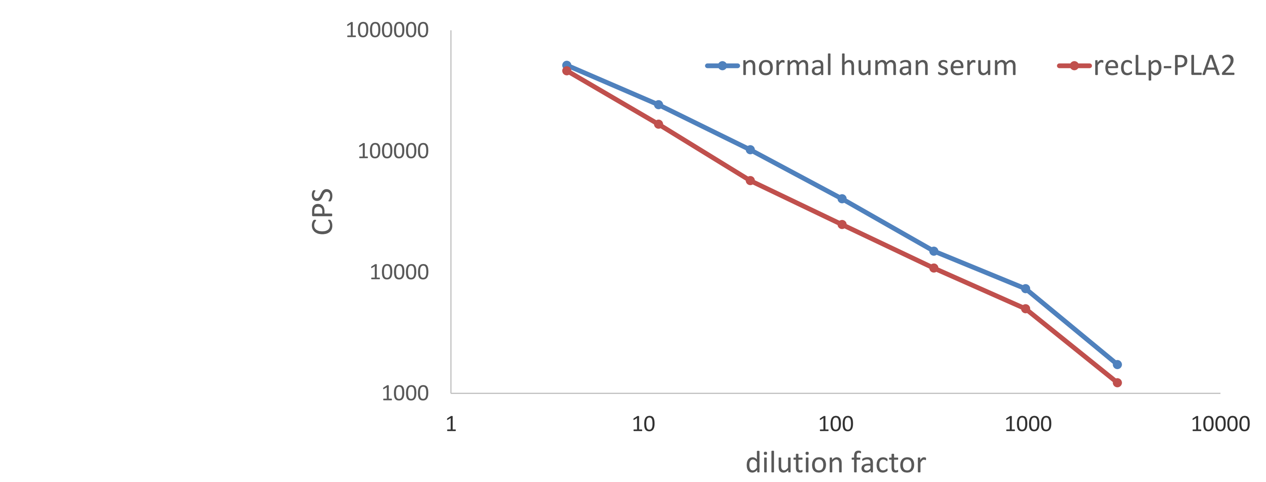

FIGURE 3. Dilutional linearity study. Recombinant Lp-PLA2 and normal human serum were titrated in a sandwich ELISA using the assay PL42-PL46. The capture antibody PL42 was coated onto the wells of a Costar EIA/RIA plate. The plate was blocked with a buffer containing 1% casein and 0.05% Tween 20 at room temperature for 15 minutes. Recombinant human Lp-PLA2 or normal human serum in serial dilutions and the detection MAb PL46 labelled with europium chelate were diluted in an assay buffer and incubated in coated plate wells for 2.5 hours at 37°C.

We determined the mass concentration of native Lp-PLA2 in the serum samples of acute myocardial infarction patients as well as in serum samples from healthy volunteers. Purified recombinant human Lp-PLA2 expressed in mammalian cell line was used as a calibrator for quantitation. There was a statistically significant difference in Lp-PLA2 mass concentration between AMI patients and healthy controls determined with the MAb combination PL26-PL4 and no significant difference for the MAb combination PL42-PL46 (see Figure 4, A and B). Although contradictory, our results are underpinned by the fact that native Lp-PLA2 in the bloodstream is a heterogeneous protein (2) and antibodies might react with its different subfractions. It should also be noted that Lp-PLA2 is primarily utilized in clinical settings as a prognostic marker of future adverse coronary events. Some authors - much like us - observed a slight increase in Lp-PLA2 mass concentration in patients with AMI compared with healthy controls (3, 4).

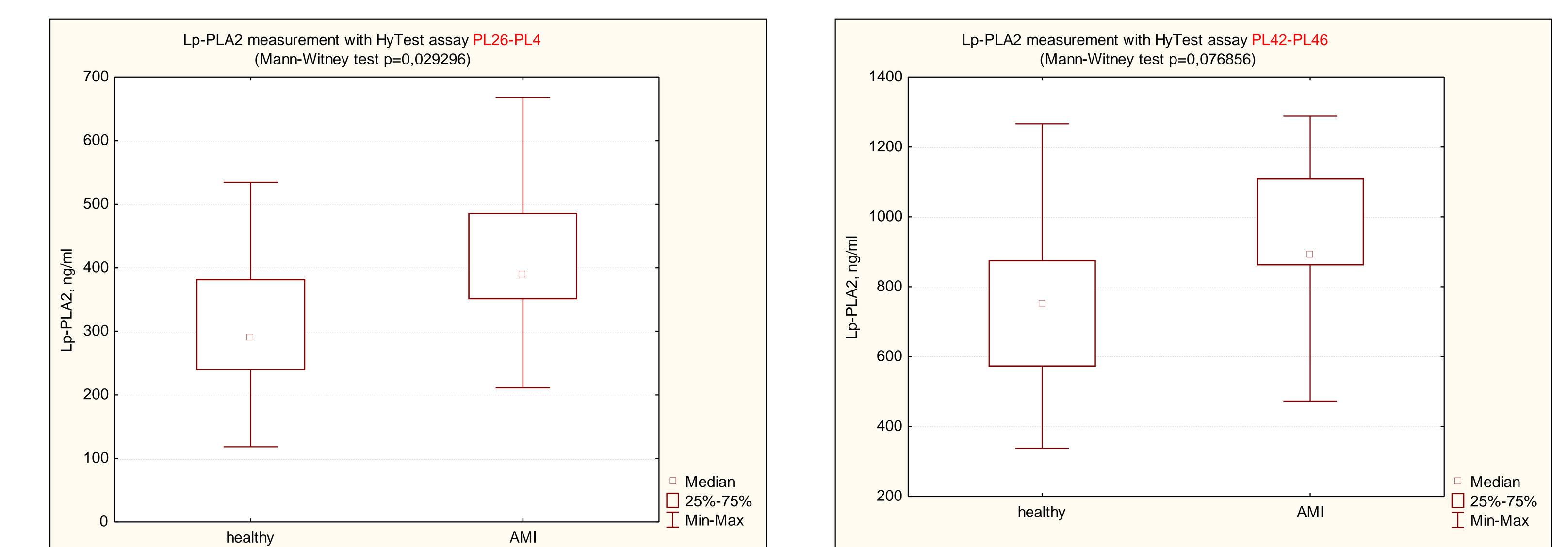

FIGURE 4. The detection of Lp-PLA2 levels in the serum of acute myocardial infarction patients and in healthy controls using the MAb combinations PL26-PL4 (A) or PL42-PL46 (B). The capture antibody PL42 or PL26 was coated onto the wells of a Costar EIA/RIA plate. The plate was blocked with a buffer containing 1% casein and 0.05% Tween 20 at room temperature for 15 minutes. Recombinant human Lp-PLA2 or serums diluted 1:30 with an assay buffer and the detection MAb PL46 or PL4 labelled with europium chelate were added and incubated in coated plate wells for 2.5 hours at 37°C.

Conclusions

1. **Anti-human Lp-PLA2 monoclonal antibodies developed in this study could be used for the development of sandwich immunoassays for quantitative measurement of Lp-PLA2.**
2. **Recombinant human Lp-PLA2 has immunochemical properties similar to that of native human Lp-PLA2 and could serve as a calibrator for immunoassays.**
3. **Prototype sandwich fluoroimmunoassay could be used for Lp-PLA2 mass concentration determination in clinical setting.**

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