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Opposite effects of native and oxidized lipoproteins on the activity of secretory phospholipase A_2 group IIA

Aleksandra A. Korotaeva*, Elena V. Samoilova, Aleksandra A. Pirkova, Vera A. Ameliushkina, Nina V. Prokazova, Vsevolod A. Tkachuk, Eugeny I. Chazov

Russian Cardiology Research Center, 3rd Cherepkovskaya str., 15A, Moscow 121552, Russia

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ABSTRACT

Elevated circulating level and activity of secretory phospholipase A_2 group IIA ($sPLA_2(IIA)$) are associated with the development of adverse cardiovascular events. The mechanisms of $sPLA_2(IIA)$ activity regulation in human blood serum so far remain obscure. We have suggested that the enzyme activity is influenced by circulating lipoproteins. The activity of $sPLA_2(IIA)$ was examined in whole serum of healthy individuals and after removal of lipoproteins from it. The effects of different classes of native and oxidized lipoproteins on $sPLA_2(IIA)$ in blood serum were compared with their effects on purified $sPLA_2(IIA)$. Activity of $sPLA_2(IIA)$ was not detected in whole serum despite the high concentration of the enzyme. However after lipoproteins had been removed from the serum, the lipoprotein-depleted serum displayed $sPLA_2(IIA)$ activity which was proportional to the amount of $sPLA_2(IIA)$ in it. Native LDL, HDL and VLDL + IDL inhibited the activity of both purified $sPLA_2(IIA)$ and the enzyme activity in lipoprotein-depleted serum. By contrast, oxidized LDL, HDL and VLDL + IDL significantly stimulated the activity of purified and serum $sPLA_2(IIA)$ and enhanced the release of fatty acids from the substrate. The data indicate that native and oxidized lipoproteins regulate catalytic activity of $sPLA_2(IIA)$. Activation of $sPLA_2(IIA)$ by oxidized lipoproteins may be regarded as one of the mechanisms of atherosclerosis development.
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1. Introduction

Group IIA secretory phospholipase A_2 ($sPLA_2(IIA)$) plays an important role in various physiological and pathophysiological processes. Activation of $sPLA_2(IIA)$ is necessary in infections, since the enzyme destroys bacterial membranes, thus protecting the organism against pathogenic microbes [1]. At the early stages of inflammation or in trauma, catalytically active $sPLA_2(IIA)$ participates in removal of injured and apoptotic cells and in formation of lipid mediators, thus facilitating activation of the defence mechanisms in the organism [2]. However, in prolonged chronic inflammation $sPLA_2(IIA)$ activation promotes the disease progression. $SPLA_2(IIA)$ stimulates angiogenesis [3], facilitates tumour growth [4], and contributes to atherogenesis [5–7] and the development of atherosclerotic lesions [8,9]. Increased serum $sPLA_2(IIA)$ activity is associated with considerably impaired endothelial vasodilator function in patients with coronary artery disease [10].

$sPLA_2(II)$ is more abundantly present in atherosclerotic culprit lesions which may cause myocardial infarction [11]. A single determination of $sPLA_2$ activity within two days after the onset of acute coronary syndrome is an independent predictor of death and new or recurrent myocardial infarction and provides a better prognostic value than the measurement of $sPLA_2(IIA)$ concentration [12]. Furthermore, the EPIC-Norfolk prospective population study has shown that catalytic activity of $sPLA_2(IIA)$ has a prognostic utility for cardiovascular diseases not only in patients but also in healthy men and women [13]. Our previous findings indicate that the development of restenosis in coronary arteries correlates better with catalytic activity than with serum content of $sPLA_2(IIA)$ in patients with coronary artery disease [14]. In some patients without restenosis serum content of $sPLA_2(IIA)$ after angioplasty was high, while $sPLA_2$ activity, in contrast, was very low. We have suggested that an inhibitor of $sPLA_2(IIA)$ which may lose its inhibitory activity under certain physiological conditions is present in human blood serum.

In this study we tested the $sPLA_2$ activity in whole and lipoprotein-depleted serum. We also examined effects of native lipoproteins (HDL, LDL and VLDL + IDL) and autooxidized lipoproteins (oxHDL, oxLDL and ox(VLDL + IDL)) on serum $sPLA_2$ activity in healthy individuals and on the activity of $sPLA_2(IIA)$ from human cardiac myxoma. The results indicate that native circulating lipoproteins inhibit $sPLA_2$ activity, whereas modified oxidized lipoproteins formed due to inflammation activate $sPLA_2(IIA)$.

* Corresponding author. Tel.: +7 495 414 6714; fax: +7 495 414 6719.
E-mail address: A.Korot@cardio.ru (A.A. Korotaeva).

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2. Materials and methods

2.1. Materials

sPLA$_2$(IIA) from human cardiac myxoma was kindly supplied by Dr. R.Sh. Bibilashvili and Dr. G.L. Khaspekov, Department of Genetic Engineering, Russian Cardiology Research Center, Moscow, Russia. The specific activity of the enzyme was 36 μmol PE hydrolyzed min^{-1} mg^{-1}. L-3-Phosphatidylethanolamine,1-acyl-2-(1-^{14}C)arachidonyl ([^{14}C]arachidonyl-PE) was purchased from Amersham Biosciences, L-α-phosphatidylcholine, dipalmitoyl (PC) and arachidonic acid were from Sigma chemical company. Thin-layer chromatography DC silica gel plates were obtained from Merk. Scintillation fluid (Unisolve 100) was from Koch-Light Ltd., England.

2.2. Preparations of whole and lipoprotein-depleted serum

Peripheral venous blood of healthy individuals was drawn in tubes without anticoagulant and centrifuged at 3000 rpm at 4 °C for 20 min. The resulting whole serum was aliquoted and stored at −70 °C until analysis. Lipoprotein-depleted serum was prepared by removing all lipoproteins from the resulting whole serum as described below. Before experiments lipoprotein-depleted serum was dialyzed against 0.01 M PBS, pH 7.4. For a relevant comparison whole serum was dialyzed under the same conditions. Lipoprotein absence and purity of lipoprotein-depleted serum were tested by cellulose acetate electrophoresis in a Scanion system (Hospitex diagnostics S.A.).

2.3. Isolation of lipoproteins

VLDL + IDL (d = 1.006–1.019 g/ml), LDL (d = 1.019–1.063 g/ml) and HDL (d = 1.063–1.215 g/ml) were isolated from blood plasma of healthy subjects by sequential ultracentrifugation in NaBr gradient and in the presence of 1 mg/ml EDTA as described previously [15]. Before analysis the lipoproteins were dialyzed against 0.01 M PBS, pH 7.4. Protein concentration was determined by the method of Lowry [16]. All lipoproteins were used within a week after isolation.

2.4. Preparation of oxidized lipoproteins

To prepare autooxidized lipoproteins freshly isolated VLDL + IDL, LDL and HDL were dialyzed against 0.01 M PBS and stored overnight at 37 °C. Oxidation of lipoproteins was estimated spectrophotometrically as production of conjugated dienes by continuously monitoring change in absorbance at 234 nm as described [17].

2.5. Preparation of [^{14}C]-labeled LDL

0.5 μCi [^{14}C]arachidonyl-PE was dissolved in chloroform, dried under a stream of nitrogen, added to 50 μl PBS and sonicated (50 s pulses) in a sonicator (model 450, Branson Ultrasonic Corp.). Freshly isolated LDL (500 μg protein) were added to the obtained suspension and incubated for 3 h at 37 °C with constant stirring. LDL was separated from unincorporated [^{14}C]arachidonyl-PE by centrifugation at a density of 1.006 g/ml for 8 h at 35,000 rpm in a Type 65 rotor (Beckman Instruments, Mountain View, CA, USA). The supernatant containing free radiolabeled PE was removed and LDL was harvested from the bottom of the tube. Approximately 90% of the [^{14}C]arachidonyl-PE was incorporated in LDL. Before analysis [^{14}C]-labeled LDL was dialyzed against PBS, pH 7.4 and protein concentration in them was determined by the method of Lowry.

2.6. Preparation of liposomes

[^{14}C-PE]-labeled liposomes were prepared with the use of [^{14}C]arachidonyl-PE (59 mCi/mmol) and PC. Radiolabeled PE (5 nmol per sample) and PC (7.5 nmol per sample) were mixed in chloroform, dried under a stream of nitrogen, dissolved in 1 ml diethyl ether and dried under nitrogen again. Dried lipids were reconstituted in the definite volume of Tris–HCl buffer (100 mM Tris, 2 mM $CaCl_2$, 0.15 M NaCl, pH 8.0) and sonicated three times for 1 min at 4 °C in a sonicator (model 450, Branson Ultrasonic Corp.). PC liposomes were prepared using only PC (the amounts are indicated).

2.7. Determination of serum sPLA$_2$(IIA) concentration

The concentration of sPLA$_2$(IIA) in whole and lipoprotein-depleted serum was determined with a monoclonal antibody using a sPLA$_2$(IIA) (human synovial) enzyme immunoassay kit from Cayman Chemical Company (Ann Arbor, MI, USA). These antibodies are specific for sPLA$_2$(IIA) and have no cross-reactivity with type I, type IV and type V sPLA$_2$. The minimum measured concentration of the enzyme was 15.6 pg/ml.

2.8. Assay of sPLA$_2$ activity

For assay of sPLA$_2$ activity various amounts of sPLA$_2$(IIA) (25–200 pg) contained in whole or lipoprotein-depleted serum were added to 10 μl [^{14}C-PE]-liposomes, 100 mM Tris–HCl, pH 8.0, 2 mM $CaCl_2$ and 0.15 M NaCl. Final volume of the reaction mixture was 500 μl. To estimate the effects of lipoproteins on sPLA$_2$(IIA) activity, indicated amounts of VLDL + IDL, LDL, HDL, or ox(VLDL + IDL), oxLDL, oxHDL were incubated with sPLA$_2$(IIA) contained in lipoprotein-depleted serum or purified sPLA$_2$(IIA) with equal activity (both enzymes hydrolyzed 30 pmol PE min^{-1}), 10 μl [^{14}C-PE]-liposomes, 100 mM Tris–HCl, pH 8.0, 2 mM $CaCl_2$ and 0.15 M NaCl in final volume of 500 μl. Indicated amounts of PC contained in liposomes were added in the control instead of lipoproteins. All reactions were carried out for 30 min at 37 °C with constant stirring and stopped by adding 1.5 ml chloroform:methanol (2:1, v/v). The upper water–methanol layer was removed. Lipids extracted in the lower chloroform layer were dried under a stream of nitrogen, dissolved in 80 μl chloroform and separated by thin-layer chromatography on silica gel plates using hexane:diethyl ether:acetic acid (85:15:1, v/v/v) solvent system. Purified arachidonic acid was used as a standard in each run. Lipid spots were visualized with iodine vapour, and fractions corresponding to free fatty acids were scraped into vials with 7 ml scintillation fluid. Radioactivity was quantified in a liquid scintillation counter. Catalytic activity was expressed as percentage hydrolyzed labeled PE relative to the control samples which were incubated in the absence of serums or lipoproteins.

3. Results

3.1. sPLA$_2$ activity in whole and lipoprotein-depleted serum

To test whether serum lipoproteins are involved in regulation of sPLA$_2$(IIA) activity we measured sPLA$_2$(IIA) activity in whole serum of healthy individuals and after removal of all lipoproteins from it. For this purpose we first determined the concentration of sPLA$_2$(IIA) in whole and lipoprotein-depleted serum and then compared the sPLA$_2$(IIA) activity in the serum aliquots containing equal amounts of the enzyme. sPLA$_2$(IIA) activity was examined by incubation of the serums with radiolabeled PE liposomes as a substrate.

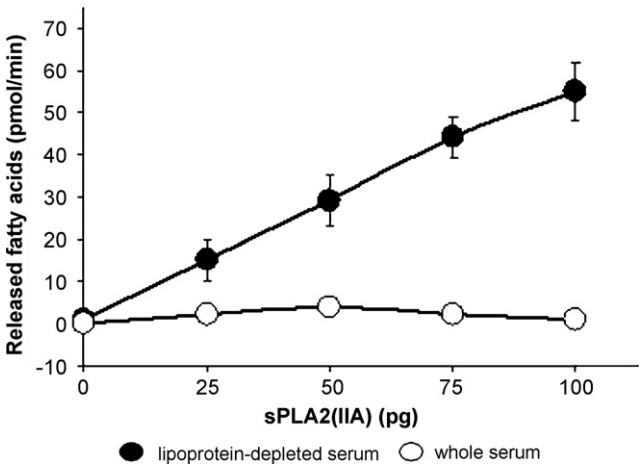

Fig. 1. $sPLA_2$(IIA) activity in whole and lipoprotein-depleted serum. Activity of $sPLA_2$(IIA) was measured in whole serum of healthy individuals (closed circles) and after removal of lipoproteins from the serum (open circles). Samples of whole and lipoprotein-depleted serum had equal contents of $sPLA_2$(IIA) indicated in the plot and were incubated for 30 min at 37 °C with liposomes containing radiolabeled PE as a substrate. The activity was evaluated from the amount of released fatty acids (mean ± SD, $n = 8$).

$sPLA_2$(IIA) contained in whole serum did not catalyze the release of fatty acids in spite of significant increase in the enzyme concentration in the incubation mixture (from 25 to 100 pg per sample) (Fig. 1). However after lipoproteins had been removed from the serum, the lipoprotein-depleted serum began to show $sPLA_2$(IIA) activity and induced release of free fatty acids from the radiolabeled substrate. As shown in Fig. 1 the activity of $sPLA_2$(IIA) increased proportionally to $sPLA_2$(IIA) content.

3.2. Effects of native HDL, LDL and VLDL + IDL on the activity of purified and serum $sPLA_2$(IIA)

In order to obtain more details about the inhibitory effect of lipoproteins we examined the influence of individual classes of lipoproteins on the activity of purified $sPLA_2$(IIA) from human cardiac myxoma. This enzyme is identical to $sPLA_2$(IIA) circulating in human blood serum [18]. The enzyme activity recorded in the assay was 30 pmol hydrolyzed PE a min. Freshly isolated LDL inhibited $sPLA_2$(IIA)-induced release of arachidonic acid from radiolabeled PE liposomes. Complete suppression of $sPLA_2$(IIA) activity was observed at LDL concentration of 60 μg protein (Fig. 2A). Native HDL and VLDL + IDL also reduced activity of $sPLA_2$(IIA) in a dose-dependent manner and completely blocked the enzyme at the concentrations of 80 and 40 μg protein, respectively. In control experiment $sPLA_2$(IIA) was incubated with radiolabeled LDL without addition of liposomes. There was no liberation of arachidonic acid from radiolabeled LDL (data not shown).

Liposomal PE is a predominant substrate for $sPLA_2$(IIA), being preferably hydrolyzed by the enzyme in comparison with PC containing in LDL. However, phospholipid exchange between liposomes and lipoproteins may occur in the incubation medium, and an increase in the amount of LDL may cause dissolution of liposomal PE by PC from LDL. As a result, the amount of hydrolyzed PE and cleaved fatty acids may decrease. In order to find out whether the reduced release of radiolabeled fatty acid is related to substrate dissolution we replaced LDL in the incubation medium with liposomes whose PC concentration was maintained within the physiological range of PC in LDL [19]. An increase in PC concentration had no effect on PE hydrolysis by $sPLA_2$(IIA), and the amount of radiolabeled arachidonic acid cleaved from PE did not change when the amount of PC in liposomes increased (Fig. 2A). Thus, our findings indicate that a dose-dependent decrease in $sPLA_2$(IIA) activity results from inhibition of substrate hydrolysis by native LDL, but not from substrate dissolution.

In order to find out whether native lipoproteins inhibit serum $sPLA_2$(IIA), we added varied amounts of freshly isolated HDL, LDL and VLDL + IDL to lipoprotein-depleted serum whose $sPLA_2$(IIA) activity was equal to the activity of purified $sPLA_2$(IIA) (30 pmol hydrolyzed PE min^{-1}). Fig. 2B shows that native HDL, LDL and VLDL + IDL reserved their inhibitory effects and suppressed $sPLA_2$(IIA) activity in lipoprotein-depleted serum.

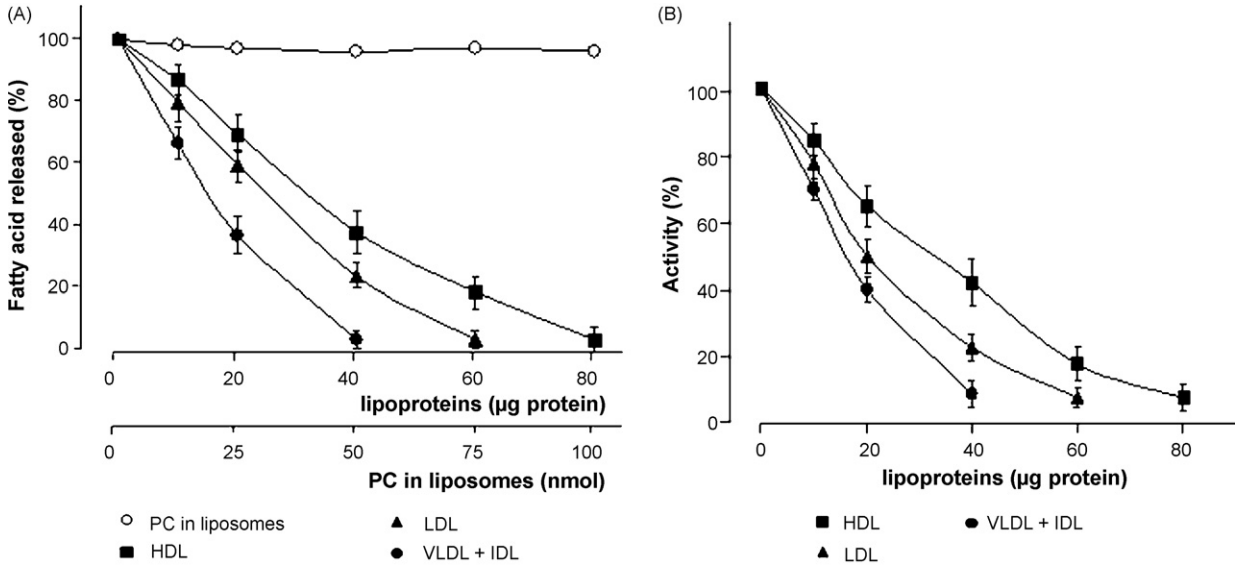

Fig. 2. Inhibition of $sPLA_2$(IIA) activity by native HDL, LDL and VLDL + IDL. (A) Purified $sPLA_2$(IIA) (the activity in the assay was 30 pmol PE hydrolyzed/min) was incubated at 37 °C for 30 min with indicated amounts of freshly isolated HDL (closed squares), LDL (closed triangles) and VLDL + IDL (closed circles). The amount of released fatty acid was determined as described in Section 2. To rule out the effect of dissolution of radiolabeled substrate by PC from lipoproteins indicated amounts of PC contained in liposomes (open circles) were added to the incubation medium instead of lipoproteins. There was no inhibition of fatty acid release in this case. (B) $sPLA_2$(IIA) in lipoprotein-depleted serum (the enzyme activity in the assay was 30 pmol PE hydrolyzed/min) was incubated with varied amounts of HDL (squares), LDL (triangles) and VLDL + IDL (circles). Incubation conditions were the same as in item A. Data were expressed as percent hydrolyzed labeled PE relative to baseline value, which was obtained in the absence of either lipoproteins or PC liposomes and set on 100% (mean ± SD, $n = 5$).

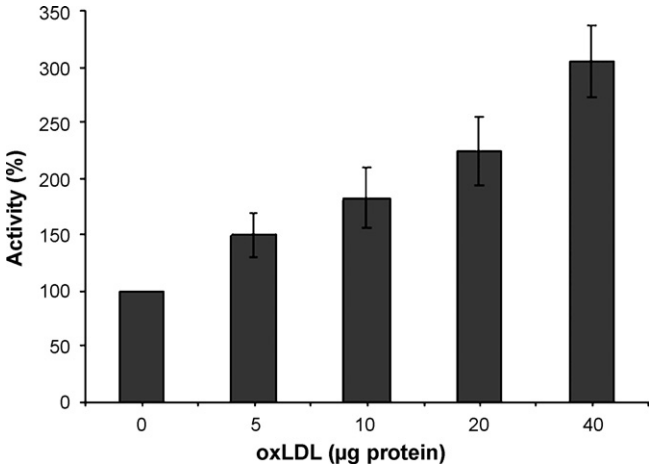

Fig. 3. Effects of oxLDL on purified $sPLA_2$(IIA) activity. OxLDL (5–40 μg protein) was added to the incubation mixture with purified $sPLA_2$(IIA) as described in Fig. 2. The $sPLA_2$(IIA) activity was expressed as percent of the activity obtained in the absence of oxLDL (100%). Data are given as mean ± SD of 5 experiments.

3.3. *Effects of oxLDL, oxHDL and ox(VLDL + IDL) on $sPLA_2$(IIA) activity*

Oxidized lipoproteins with properties different from those of native lipoproteins are formed in inflammation. We have suggested that the effect of lipoproteins on $sPLA_2$(IIA) activity is modified by their oxidation. To test this suggestion we prepared oxHDL, oxLDL and ox(VLDL + IDL) (as described in Section 2) and incubated them with purified $sPLA_2$(IIA) or $sPLA_2$(IIA) in lipoprotein-depleted serum. As shown in Fig. 3, oxLDL significantly stimulated activity of purified $sPLA_2$(IIA). An increase in the amount of oxLDL induced a considerable rise in the amount of fatty acids released from the labeled substrate. Approximately 3-fold increase in the activity (relative to basal activity) was observed upon the addition of oxLDL at the concentration of 40 μg protein ($p < 0.01$). A similar effect was produced by oxHDL and ox(VLDL + IDL) on purified $sPLA_2$(IIA) (data not shown).

Fig. 4 shows that oxLDL, oxHDL and ox(VLDL + IDL) also markedly stimulated activity of $sPLA_2$(IIA) in lipoprotein-depleted serum.

To find out whether LDL-associated PAF–acylhydrolase or HDL-associated lecithin:cholesterol acyltransferase contributes to the increase in the amount of released arachidonic acid, oxLDL or oxHDL were incubated with radiolabeled PE liposomes. The enzymes did not hydrolyze PE liposomes (data not shown). Thus, the increase in the amount of hydrolyzed product results from stimulation of $sPLA_2$(IIA) activity by oxidized lipoproteins.

4. Discussion

In this study we have shown for the first time that serum lipoproteins regulate $sPLA_2$(IIA). Regulation of $sPLA_2$(IIA) has great physiological and clinical significance. Activation of $sPLA_2$(IIA) leads to hydrolysis of cell membrane phospholipids and lipoproteins. Among the products of this reaction are free fatty acids, the precursors of inflammation lipid mediators, leukotrienes, prostaglandins and other eicosanoids that initiate inflammation and aggravate unstable atherosclerotic plaques [20]. Bioactive lysophospholipids which play a significant role in the development of cardiovascular diseases are another product of $sPLA_2$(IIA) reaction. It was demonstrated that lysophosphatidylcholine contributes to atherosclerosis and ischemia by impairing the endothelium-dependent vascular relaxation, modulating contraction of smooth muscle cells, increasing proliferation and permeability of endothelial cells, stimulating leukocyte adhesion and activation, initiating macrophage chemotaxis, and modifying platelet aggregation [21,22].

$sPLA_2$(IIA) is expressed in peripheral blood as a mature catalytically active protein [23] capable of hydrolyzing the ester bond in phospholipids. Millimolar concentrations of calcium ions and neutral pH of blood serum are optimal for catalytic activity of $sPLA_2$(IIA). However, activity of the enzyme is not always detected in blood serum. In the present study we did not detect the activity of $sPLA_2$(IIA) in whole serum of healthy individuals irrespective of high contents of the enzyme. The activity, however, was detected after removal of lipoproteins from the serum. These results suggest the presence of $sPLA_2$(IIA) inhibitor in whole serum which is removed together with lipoproteins. Exact mechanisms of $sPLA_2$(IIA) inactivation in human blood serum are presently unknown. Protein which selectively inhibits $sPLA_2$(IIA) in human blood serum so far has not been identified, while the protein from snake and bee venom have been isolated and characterized.

We have found that native HDL, LDL and VLDL + IDL suppress the activity of $sPLA_2$(IIA). These data suggest that lipids of a lipoprotein particle act as $sPLA_2$(IIA) inhibitors in human blood. It was demonstrated in vitro that some sphingolipids inhibit or stimulate $sPLA_2$(IIA) activity [24,25].

Small amounts of other secretory phospholipases A_2 such as group V and group X may be present at blood. Since these enzymes are minor in comparison with $sPLA_2$(IIA), they exert no significant effect on serum $sPLA_2$ activity. Moreover, since $sPLA_2$ activity was completely inhibited, the results obtained allow one to suggest that similar secretory phospholipases are also inhibited by native lipoproteins. This suggestion is supported by the results of other researchers, indicating that both group V secretory phospholipases A_2 and $sPLA_2$(IIA) are inhibited by sphingomyelin [26].

In inflammatory processes oxidized lipoproteins are formed in blood serum. It is well known that oxLDL is considered as the major cause of atherosclerosis [27]. $sPLA_2$(IIA) is also expressed during inflammation and involved in all stages of atherosclerotic lesion development [7,9]. A strong correlation between the levels of $sPLA_2$(IIA) and oxLDL was demonstrated in clinical studies [28]. The results presented here have shown that oxidized lipopro-

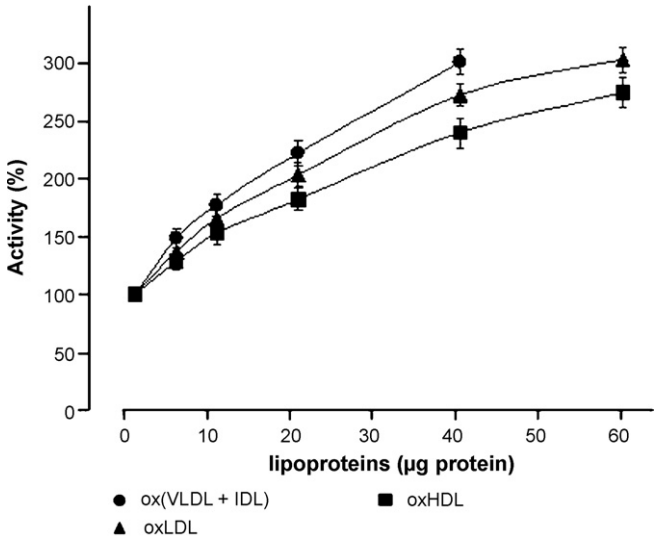

Fig. 4. Stimulation of $sPLA_2$(IIA) activity in lipoprotein-depleted serum by oxHDL, oxLDL and ox(VLDL + IDL). Different amounts of oxHDL (squares), oxLDL (triangles) and ox(VLDL + IDL) (circles) were incubated with $sPLA_2$(IIA) in lipoprotein-depleted serum. Incubation conditions were the same as described in Fig. 2. The activity was calculated as percent of released fatty acids obtained in the absence of oxidized lipoproteins (100%). Values are means ± SD of five independent experiments.

teins (oxHDL, oxLDL and ox(VLDL + IDL)) considerably stimulate the $sPLA_2$(IIA) activity. This may represent one of the mechanisms of $sPLA_2$(IIA) activation in inflammation and atherosclerosis. Biologically active oxidized phospholipids and lysophosphatidylcholine form and accumulate upon oxidation of lipoproteins. It can be suggested that the observed stimulatory effect of oxidized lipoproteins on $sPLA_2$(IIA) is associated with changes in their phospholipid composition.

Control over the mechanisms of $sPLA_2$(IIA) regulation could be a promising strategy in cardiovascular therapy [29]. Pharmacological inhibition of $sPLA_2$ activity was used in clinical practice. It has been shown that statins and angiotensin II type 1 receptor blockade reduce $sPLA_2$(IIA) activity and oxLDL level in patients with coronary artery disease [30]. Our previous studies have shown that atorvastatin decreases the content and activity of $sPLA_2$(IIA) in patients with initially increased serum enzyme content [31]. In guinea pigs inhibition of $sPLA_2$(IIA) by varespladib reduced athoresclerotic lesions by 24% [32]. There were attempts to use synthetic inhibitors of $sPLA_2$(IIA) in acute inflammatory diseases such as severe sepsis and rheumatoid arthritis in which the enzyme is expressed in great amounts, however, these studies showed no positive results [33,34]. Since $sPLA_2$(IIA) is physiologically expressed in inflammation, the enzyme inhibition may have clinical effect not in acute but in chromic inflammation, such as atherosclerosis.

The results obtained in this study demonstrate a new function of circulating native and oxidized lipoproteins formed during inflammation in the regulation of proinflammatory $sPLA_2$(IIA) activity in human blood serum and can be used for further investigation of $sPLA_2$(IIA) regulatory components of lipoprotein particles.

Acknowledgments

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