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LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A_2 IS INCREASED IN PATIENTS WITH IMPAIRED BONE DENSITY

FOSFOLIPAZA A_2 UDRUŽENA SA LIPOPROTEINOM JE POVIŠENA KOD PACIJENATA SA SMANJENOM GUSTINOM KOSTIJU

Karel Kotaška[1], Jitka Kolářová[1], Blanka Jedličková[1], Jana Čepová[1], Jan Kotaška[2], Richard Průša[1]

[1]Department of Medical Chemistry and Clinical Biochemistry, 2nd Faculty of Medicine, Charles University, Faculty Hospital Motol, Prague, Czech Republic
[2]Department of Orthopaedics, 2nd Faculty of Medicine, Charles University, Faculty Hospital Motol, Prague, Czech Republic

Summary

Background: Increased levels of lipoprotein-associated phospholipase A_2 are associated with atherosclerosis, and may contribute to cardiac disease. The aim of this study was to analyze serum levels of lipoprotein phospholipase A_2 (Lp-PLA_2) in patients with impaired bone resorption and correlate the findings with markers of bone metabolism (osteocalcin) and other biochemical markers (cholesterol, low density lipoprotein, triacylglycerols).
Methods: Serum Lp-PLA_2 was measured by a turbidimetric method in a group of currently treated 85 patients with impaired bone resorption and in a control group of 46 healthy individuals. Serum triacylglycerols was measured by the electrochemiluminescence immunoassay. Cholesterol, low density lipoprotein and triacylglycerols were measured by commercially available enzymatic assays. Bone density was investigated by dual energy X-ray densitometry performed on the lower spine and hips.
Results: Concentrations of LP-PLA_2 were significantly elevated in the patients with bone resorption compared to the control group of healthy individuals (225 ng/mL vs. 192 ng/mL, $p<0.001$) with the highest difference in patients with a T score below –2.5 SD (227 vs. 192 ng/mL). Serum

Kratak sadržaj

Uvod: Povišeni nivoi fosfolipaze A_2 udružene sa lipoproteinom povezani su sa aterosklerozom i mogu doprineti razvoju srčanih oboljenja. Cilj ove studije bio je da se analiziraju nivoi fosfolipaze A_2 udružene sa lipoproteinom (Lp-PLA_2) u serumu pacijenata sa povećanom koštanom resorpcijom i napravi korelacija dobijenih nalaza sa markerima koštanog metabolizma (osteokalcin) i drugim biohemijskim markerima (holesterol, lipoprotein male gustine, triacilgliceroli).
Metode: Lp-PA_2 u serumu meren je metodom turbidimetrije u grupi od 85 pacijenata na terapiji zbog povećane koštane resorpcije i u kontrolnoj grupi sa 46 zdravih osoba. Osteokalcin u serumu meren je elektrohemiluminiscentnim imunoesejom. Holesterol, lipoprotein male gustine i trigliceridi izmereni su komercijalnim enzimatskim testovima. Gustina kostiju je ispitana tehnikom *dual energy X-ray* denzitometrije primenjenom na donji deo kičmenog stuba i kukove.
Rezultati: Koncentracije Lp-PLA_2 bile su značajno povišene kod pacijenata sa koštanom resorpcijom u poređenju s kontrolnom grupom zdravih osoba (225 ng/mL vs. 192 ng/mL, $p<0,001$), a najveća razlika je zabeležena kod pacijenata sa T-skorom nižim od –2,5 SD (227 vs. 192 ng/mL). Nivoi Lp-

Address for correspondence:

Karel Kotaška, MSc., Ph.D.
Dept. of Medical Chemistry and Clinical Biochemistry
2nd Faculty of Medicine, Charles University
Faculty Hospital Motol
V uvalu 84,
150 06 Prague 5
Tel: +420224435341
Fax: +420224435320
e-mail: kotaska@email.cz

List of abbreviations: Lp-PLA_2, lipoprotein-associated phospholipase A_2; PLA_2, phospholipase A_2; DXA, dual-energy x-ray absorptiometry; PAF-AH, platelet activating factor acetylhydroxylase; LDL, low density lipoprotein; SD, standard deviation; OR, ODDs ratio; PAF, platelet activating factor; ECLIA, electrochemiluminescence immunoassay.

levels of Lp-PLA_2 also negatively correlated with decreased levels of serum osteocalcin in patients, and a significant difference in Lp-PLA_2 ($p=0.02$) levels was observed between the control group and group with low levels of osteocalcin. Elevated Lp-PLA_2 levels were significantly associated with the therapeutic procedures used, but not with age, gender and concentration of lipids.
Conclusions: Lipoprotein-associated phospholipase A_2 seems to play an important role also in bone metabolism.

Keywords: lipoprotein-associated phospholipase A_2, osteocalcin, bone metabolism

PLA_2 u serumu također su bili u negativnoj korelaciji sa smanjenim nivoima osteokalcina u serumu pacijenata, dok je između kontrolne grupe i grupe sa niskim nivoima osteokalcina uočena značajna razlika u nivou Lp-PLA_2 ($p=0,02$). Povišeni nivoi Lp-PLA_2 bili su u značajnoj asocijaciji sa korišćenim terapijskim postupcima, ali ne i sa starošću, polom i koncentracijom lipida.
Zaključak: Fosfolipaza A_2 udružena sa lipoproteinom izgleda igra važnu ulogu i u metabolizmu kostiju.

Ključne reči: fosfolipaza A_2 udružena sa lipoproteinom, osteokalcin, metabolizam kostiju

Introduction

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a circulating enzyme belonging to the unrelated phospholipase A_2 protein families with common enzymatic activity. The two most notable families are secreted phospholipases A_2 and cytosolic phospholipases A_2. Other families include Ca^{2+} independent PLA_2 (iPLA_2) and lipoprotein-associated PLA_2, also known as platelet activating factor acetylhydrolase (PAF-AH). Recent evidence has established physiological and pathological roles of PLA_2 enzymes in fertility, muscle growth, renal concentration, post-ischemic brain injury, inflammatory and oxidative activities associated with cardiovascular disease and ischemic stroke, inflammatory bone resorption, intestinal polyposis, pulmonary fibrosis, acute respiratory distress syndrome and autoimmune encephalomyelitis (1).

Cytosolic phospholipases preferentially hydrolyze phospholipids containing arachidonic acid and play a key role in the biosynthesis of eicosanoids including prostaglandins and leucotrienes (2). Hydrolysis leads to release of free arachidonic acid which in turn is metabolized to prostaglandins. Prostaglandin (especially prostaglandin E_2) is produced by osteoblasts and acts as a potent stimulator of bone resorption (1, 3).

The Lp-PLA_2 is platelet-activating factor (PAF) acetylhydrolase (EC 3.1.1.47) catalyzing the degradation of PAF to inactive products by hydrolysis of the acetyl group at the sn-2 position, and thus producing the biologically inactive products LYSO-PAF and acetate (4). Lp-PLA_2 shows mainly proinflammatory and oxidative activities preferably associated with cardiovascular disease (5). The association of Lp-PLA_2 with bone resorption is not yet well known, and thus it seems interesting to analyze the serum levels of Lp-PLA_2 in patients with impaired bone density.

Materials and Methods

Patients

Eighty-five patients with various levels of bone density (4 males, mean age 56 years, and 81 postmenopausal women, mean age 70 years) were enrolled in the study. The patients were previously clinically classified for bone mineral density. According to the recent bone density guidelines, the patients were divided into three groups. Group I consists of 43 patients with osteoporosis, group II consists of 20 patients with osteopenia and group III consists of 22 patients with normal bone density. Fifty-seven patients were currently on combined lipid lowering and antiresorption therapy (statins and bisphosphonates), while 11 patients were already on lipid lowering therapy (statins). Seventeen patients were supplemented with vitamin D_3.

We compared the patients with a group of 46 healthy individuals (14 males, mean age 55 years, and 32 females, mean age 47 years) without any signs of bone resorption and thus without any therapy. Characteristics of the individuals and patients included in the study are listed in *Table I*.

Table I Characteristics of the subjects.

	Control group (n=46)	All patients (N=85)
Mean age (years)	55	67
Males/Females	14/32	4/81
S-Lp-PLA_2 (ng/mL)	192 (159–227)	225 (193–253) $p<0.001$
S-Osteocalcin (μg/L)	30.3 (17.6–37)	18.4 (14.2–20.6) $p<0.05$
S-LDL cholesterol (mmol/L)	3.1 (1.0)	2.9 (0.7)
S-Cholesterol (mmol/L)	5.2 (1.0)	5.1 (0.7)
S-Triacylglycerols (mmol/L)	1.34 (1.02–1.88)	1.13 (0.86–1.5) $p = 0.03$

Concentrations are expressed as mean with (SD), or median and interquartile ranges (25^{th}–5^{th} percentile).

Sample collection

Blood specimens were collected by venipuncture in vacuum collection tubes. After collection, specimens were centrifuged (4 °C at 1500 G for 10 minutes). Serum osteocalcin, total cholesterol, LDL-cholesterol levels, and triglycerides were immediately investigated.

Prior to Lp-PLA_2 testing, aliquots of serum were stored at 2–8 °C overnight and assayed on the next day after the blood collection (2nd day after the collection of blood).

Biochemical analysis

Serum levels of Lp-PLA_2 were measured under conditions specified in the manufacturer's instruction, by a commercially available turbidimetric assay for the quantitative determination of Lp-PLA_2 (PLAC Test, DiaDexus, San Francisco, USA) on an automatic biochemical analyzer Advia 1800 (Siemens). The reference values for Lp-PLA_2 provided by the manufacturer of the diagnostic kit were less than 200 ng/mL.

Expected values for serum levels of osteocalcin were assayed by the commercially available electrochemiluminescence immunoassay (ECLIA Roche, Mannheim, Germany) on an automatic analyzer COBAS e411 (Roche). Serum levels of cholesterol, LDL-cholesterol and triglycerides were assayed by the commercially available direct enzymatic assays on an automatic biochemical analyzer Advia 1800 (Siemens).

Bone density investigations

Dual-energy x-ray absorptiometry (DXA) performed on the lower spine and hips was used to evaluate the bone density. Patients were divided into three groups according to their bone density as defined by the T-score. The T-score is a person's bone mass at a particular site, expressed in standard deviations (SD) away from the mean of a reference population. T-score above –1 SD is considered normal, T-score between –1 and –2.5 SD is classified as osteopenia (low bone mass) and T-score below –2.5 SD is defined as osteoporosis.

Statistical analysis

Shapiro-Wilk normality test was used to determine the distribution of the data. One-way analysis of variance with Newmann-Keuls multiple test or unpaired T-test was used if the distribution of data was normal, and in case of non-parametric data distribution, Kruskal-Wallis test or Mann-Whitney U-test were used to evaluate the Lp-PLA_2 levels in selected groups. Fisher's exact test and odds ratio were used to investigate the association between Lp-PLA_2 and lipid parameters (total cholesterol, LDL-cholesterol) or age, sex and therapy.

A value of $p<0.05$ was considered statistically significant. Statistical software GraphPad Prism, version 6.0 (San Diego, California) was used to perform the statistical analysis.

Results

Serum levels of Lp-PLA_2 were measured in the control group of healthy individuals and in the whole group of patients. The median Lp-PLA_2 value in patients was significantly elevated in comparison with healthy individuals (225 ng/mL vs. 192 ng/mL, $p<0.001$ – Mann-Whitney U-test).

Patients were divided into three groups (osteoporosis, osteopenia and normal bone density) according to the T-score estimated from densitometry. T-score is expressed as the standard deviation of bone density from a reference population, as described previously.

Medians of Lp-PLA_2 concentrations in patients with osteoporosis, osteopenia and normal bone density according to DXA were significantly elevated, contrary to the control group, with the highest difference in patients with osteoporosis and osteopenia (227 ng/mL and 222 ng/mL vs. 192 ng/mL, $p=0.004$ and $p=0.005$ – Mann-Whitney U-test). Concentration of Lp-PLA_2 in patients with normal

Table II Concentration of serum Lp-PLA_2 (ng/mL) in healthy individuals and in patients with impaired bone density in various stages.

Group	n	Lp-PLA_2 (ng/mL)	p value
Healthy individuals	46	192 (159–227)	
All patients	85	225 (193–253)	<0.001*
T > –1	20	221 (192–251)	0.024*
T = –1 to –2.5	22	222 (198–250)	0.005*
T < –2.5	43	227 (187–263)	0.004*

Results expressed as median and interquartile range (25th and 75th percentile)

*Value of $p<0.05$ is considered as statistically significant. Differences between concentrations in patients with osteoporosis, osteopenia and normal bone density according to DXA were considered not significant ($p=0.76$ Newmann-Keuls multiple comparison test).

Table III Concentration of serum Lp-PLA2 (ng/mL) in healthy individuals and in the subgroup of patients with decreased serum osteocalcin levels below the lower limit of the reference interval.

Group	n	Lp-PLA$_2$ (ng/mL)	p value
Healthy individuals	46	192 (159–227)	
Patients	24	224 (194–264)	0.005*
T < –2.5	13	260 (214–295)	0.002*
T = –1 to –2.5	6	198 (189–218)	0.49
T > –1	5	213 (194–236)	0.24

Results expressed as median and interquartile range (25th and 75th percentile)

*Value of $p<0.05$ is considered as statistically significant. Differences between concentrations in patients with osteoporosis, osteopenia and normal bone resorption according to DXA are considered not significant ($p=0.11$, Newmann-Keuls multiple comparison test).

bone density was also increased (221 ng/mL + 33 ng/mL) and reached significant difference from the control group ($p=0.024$ – Mann-Whitney U-test).

The results are summarized in *Table II*.

Serum osteocalcin, as a marker of osteosynthesis and bone remodeling, was also measured in all the patients. The reference intervals for serum osteocalcin were as follows: males from 50 to 70 years: 14–46 μg/L, females younger than 55: 11–43 μg/L, females older than 56: 15–46 μg/L.

Serum levels of Lp-PLA$_2$ in patients with the serum osteocalcin concentration decreased below the reference interval were compared with a control group of healthy individuals. Twenty-four patients from the entire group (n=85) had a serum level of osteocalcin below the lower limit of the reference interval.

The median Lp-PLA$_2$ value in all the patients with a decreased serum osteocalcin concentration (below the lower limit of the reference interval) was significantly elevated in comparison with the healthy individuals (224 ng/mL vs. 192 ng/mL, $p=0.005$ – Mann-Whitney U-test).

In the subgroup of patients with osteoporosis and decreased serum levels of osteocalcin, the median of Lp-PLA$_2$ was significantly elevated, contrary to the control group (260 pg/mL vs. 192 pg/mL $p=0.002$ – Mann-Whitney U-test). Medians in other subgroups (osteopenia and normal density) with decreased serum levels of osteocalcin did not reach statistical significance ($p=0.49$ and $p=0.24$). The results are summarized in *Table III*.

Concentration of Lp-PLA$_2$ inversely correlated with decreased levels of serum osteocalcin (mean Lp-PLA$_2$ concentration in patients with decreased osteocalcin = 237.2 ng/mL vs. mean Lp-PLA$_2$ concentration in patients with normal osteocalcin = 220.8 ng/mL, Pearson correlation coefficient $r=-0.28$).

We investigated the association of Lp-PLA$_2$ elevation with the therapeutic procedures used (antiresorption therapy and antiresorption therapy combined with lipid lowering therapy) and both associations were found significant ($p=0.014$, odds ratio – $OR=3.36$ and $p=0.007$, $OR=2.97$ respectively).

Discussion

Our results support the evidence for a significant role of phospholipase A$_2$ in the metabolic processes of bone metabolism. It is well known that phospholipase A$_2$ is involved in the metabolism of prostaglandins (particularly prostaglandin E$_2$) acting as a stimulator of bone resorption. The lipoprotein phospholipase A$_2$ belongs to a subgroup of the Ca^{2+} independent PLA$_2$ family with a unique substrate preference for lysophospholipids, and thus is mainly involved in atherosclerotic processes. Lp-PLA$_2$ is responsible for generating two proinflammatory mediators following the oxidation of LDL: lysophosphatidylcholine and oxidized fatty acid, and thus is significantly associated with cardiovascular diseases and ischemic stroke (5). The cut off value defined in clinical guidelines for the management of cardiovascular disease is set at 200 ng/mL (6). Our results show that medians of serum Lp-PLA$_2$ in patients with impaired bone remodeling (according to DXA) ranged from 221 to 227 ng/mL, and were therefore elevated above the approved cut off value for cardiovascular disease. This finding could lead to the question of whether these patients with impaired bone metabolism are also at elevated risk of cardiovascular events. Nevertheless, patients were currently clinically investigated and treated by standard therapy (vitamin D$_3$, bisphosphonates, statins). Bisphosphonates are known as powerful inhibitors of bone resorption (7). It is well known that decreased levels of serum osteocalcin correlate with long-term bisphosphonates treatment. This decrease was cofirmed in the group of patients with impaired bone resorption, as shown in *Table I*.

We found a negative correlation of Lp-PLA$_2$ levels with decreased levels of serum osteocalcin (Pearson correlation coefficient $r=-0.28$). This suggests that Lp-PLA$_2$ might be an additional, promising biochemical marker of bone metabolism, however, this needs to be verified on a larger number of samples.

Recent data show that bisphosphonates may also provide protective effects against cardiovascular events including acute myocardial infarction (8).

Lp-PLA_2 activity correlates with lipid parameters. A strong positive correlation has been demonstrated consistently with LDL and total cholesterol in many epidemiological studies (9–15). We confirmed this correlation in our study (Pearson correlation coefficient r=0.93). We analyzed the association of lipid parameters with Lp-PLA_2 levels and we found this association not significant (p=0.64, Fischer's exact test, odds ratio – OR=0.76). This suggests that the increased concentration of Lp-PLA_2 in our group of patients with impaired bone density was independent of the levels of LDL-cholesterol and total cholesterol. Although the control group is younger than the patient group (55 vs. 67 years), there was no significant association of Lp-PLA_2 levels with age (OR=1.48, p=0.49). Lp-PLA_2 levels are also independent of gender (OR=1, p=0.67). Serum levels of Lp-PLA_2 were significantly associated with the therapeutic procedures used. We found a significant association of Lp-PLA_2 levels with combined antiresorption and with lipid lowering therapy using bisphosphonates and statins (OR=2.97, p=0.007). Relationship of Lp-PLA_2 concentrations with antiresorption therapy using bisphosphonates was also significant (OR=3.36, p=0.014). On the contrary, the association with statin therapy and vitamin D_3 supplementation was not significant (p=0.09 and p=0.15).

The use of Lp-PLA_2 as a marker of bone resorption in routine laboratory practice implies some analytical specifications including specimen handling, sample storage and the time of analysis. Although Lp-PLA_2 is transported coupling with either LDL, or HDL and lipoprotein (a), it is necessary to perform the assay after sample stabilization during 16 hours at 2–8 °C, and the assay could be performed between 16–72 hours after the blood collection when Lp-PLA_2 is eliminated from coupling with lipoproteins. Alternatively, the assay could be performed on the second day after freezing overnight at less than –20 °C.

Conclusion

Lipoprotein-associated phospholipase A_2 seems to play an important role in bone metabolism. However, more analyses need to be performed to confirm its significance.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

References

1. Hirabayashi T, Murayama T, Shimizu T. Regulatory mechanism and physiological role of cytosolic phospholipase A_2. Biol Pharm Bull 2004; 27: 1168–73.
2. Murakami M, Kudo I. Phospholipase A_2. J Biochem 2002; 131: 285–92.
3. Miyaura C, Inada M, Matsumoto C, Ohshiba T, Uozumi N, Shimizu T, et al. An essential role of cytosolic phospholipase A2alpha in prostaglandin E2-mediated bone resorption associated with inflammation. J Exp Med 2003; 197: 1303–10.
4. Tjoelker LW, Wilder C, Eberhardt C, Stafforini DM, Dietsch G, Schimpf B, et al. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. Nature 1995; 374: 549–53.
5. Davidson MH, Corson MA, Alberts MJ, Anderson JL, Gorelick PB, Jones PH, et al. Consensus panel recommendation for incorporating lipoprotein-associated phospholipase A2 testing into cardiovascular disease risk assessment guidelines. Am J Cardiol 2008; 101: Suppl. 12A: 51F-7F.
6. Caslake MJ, Packard CJ. Lipoprotein-associated phospholipase A_2 (platelet-activating factor acetylhydrolase) and cardiovascular disease. Curr Opin Lipidol 2003; 14: 347–52.
7. Fleisch H. Bisphosphonates: mechanisms of action. Endocr Rev 1998; 19: 80–100.
8. Kang JH, Keller JJ, Lin HC. Bisphosphonates reduced the risk of acute myocardial infarction: a 2-year follow-up study. Osteoporos Int 2012; 24: 271–7.
9. Hatoum IJ, Nelson JJ, Cook NR, Hu FB, Rimm EB. Dietary, lifestyle, and clinical predictors of lipoprotein-associated phospholipase A_2 activity in individuals without coronary artery disease. Am J Clin Nutr 2010; 91: 786–93.
10. Coskun A, Serteser M, Unsal I. Inhibition of cholesterol biosynthesis in hypercholesterolemia – is it the right choice? J Med Biochem 2013; 32: 16–19.
11. Pavlović D, Josipović J, Pavlović N. Vitamin D in cardiovascular and renal disease prevention. J Med Biochem 2013; 32: 11–15.
12. Holick FM. The D-lightful vitamin D for health. J Med Biochem 2013; 32: 1–10.
13. Persson M, Nilsson JA, Nelson JJ, Hedblad B, Berglund G. The epidemiology of Lp-PLA(2): distribution and correlation with cardiovascular risk factors in a population-based cohort. Atherosclerosis 2007; 190: 388–96.

14. Ballantyne CM, Hoogeveen RC, Bang H, Coresh J, Folsom AR, Heiss G, et al. Lipoprotein-associated phospholipase A_2, high-sensitivity C-reactive protein, and risk for incident coronary heart disease in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study. Circulation 2004; 109: 837–42.

15. Blake GJ, Dada N, Fox JC, Manson JE, Ridker PM. A prospective evaluation of lipoprotein-associated phospholipase A(2) levels and the risk of future cardiovascular events in women. J Am Coll Cardiol 2001; 38: 1302–6.

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