Secretory phospholipase A_2 activity is linked to hypercholesterolemia and gender in non-alcoholic fatty liver disease individuals

Związek aktywności zewnątrzwydzielniczej fosfolipazy A_2 z hipercholesterolemią oraz płcią u pacjentów z niealkoholową stłuszczeniową chorobą wątroby

Joanna Raszeja-Wyszomirska[1], Krzysztof Safranow[2], Agnieszka Szynkowska[3], Ewa Stachowska[3]

[1]Liver Unit, Pomeranian Medical University, Szczecin, Poland
[2]Department of Biochemistry, Pomeranian Medical University, Szczecin, Poland
[3]Department of Biochemistry and Human Nutrition, Pomeranian Medical University, Szczecin, Poland

Prz Gastroenterol 2013; 8 (3): 172–175
DOI: 10.5114/pg.2013.36330

Key words: secretory phospholipase A_2, isoprostane, 8-epi-$PGF_{2\alpha}$ III, hydroxyoctadecadienoic acid.
Słowa kluczowe: wydzielnicza forma fosfolipazy A_2, izoprostan, 8-epi-$PGF_{2\alpha}$ III, kwas hydroksydekadienowy.

Address for correspondence: Ewa Stachowska MD, Department of Biochemistry and Human Nutrition, Pomeranian Medical University, 24 Broniewskiego St, 71-460 Szczecin, Poland, phone: +48 91 441 48 06, fax: +48 91 441 48 07, e-mail: ewast@sci.pam.szczecin.pl

Abstract

Introduction: Non-alcoholic fatty liver disease (NAFLD) is associated with the metabolic syndrome, which is a constellation of insulin resistance, central obesity, dyslipidemia and inflammation. During the inflammatory process polyunsaturated fatty acids are converted into proinflammatory hydroperoxides by the action of lipoxygenases, e.g. secretory phospholipase A_2 ($sPLA_2$).

Aim: The role of $sPLA_2$ during progression of inflammation in NAFLD is practically unknown; therefore the aim of the study was to investigate $sPLA_2$ activity in NAFLD patients.

Material and methods: Ninety-two patients with NAFLD and 20 healthy individuals were enrolled in the study. $sPLA_2$ activity in plasma was evaluated by the ELISA method.

Results: There were no differences between NAFLD and healthy individuals in $sPLA_2$ activity but among NAFLD individuals high $sPLA_2$ activity was linked to male gender and high concentration of plasma cholesterol (hypercholesterolemia). A trend for a correlation between 8-epi-$PGF_{2\alpha}$ III concentration in plasma and $sPLA_2$ activity was observed.

Conclusions: $sPLA_2$ is one of the main factors associated with progression of inflammatory diseases including NAFLD. A positive correlation between total cholesterol in plasma and activity of $sPLA_2$ in men may contribute to the pathogenesis of NAFLD as well as progression to steatohepatitis according to novel "one hit" theory.

Streszczenie

Wstęp: Niealkoholowa stłuszczeniowa choroba wątroby (*non-alcoholic fatty liver disease* – NAFLD) jest ściśle powiązana z klinicznymi cechami zespołu metabolicznego, w skład którego wchodzą insulinooporność, otyłość trzewna, zaburzenia lipidowe oraz przewlekły proces zapalny. Proces ten wiąże się z przemianą wielonienasyconych kwasów tłuszczowych poprzez lipooksydazy w ich prozapalne pochodne wodorotlenowe, a jednym z zaangażowanych w ten proces enzymów jest wydzielnicza forma fosfolipazy A_2 ($sPLA_2$).

Cel: Ponieważ rola $sPLA_2$ w rozwoju procesu zapalnego w NAFLD nie jest znana, celem niniejszej pracy była ocena jej aktywności w NAFLD.

Materiał i metody: Do badania włączono 92 osoby z NAFLD oraz 20 osób zdrowych, u których aktywność $sPLA_2$ oceniano metodą ELISA.

Wyniki: Nie stwierdzono różnic w aktywności $sPLA_2$ pomiędzy osobami zdrowymi a pacjentami z NAFLD, ale w tej drugiej grupie wyższą aktywność $sPLA_2$ obserwowano u mężczyzn oraz u osób z hipercholesterolemią. Stwierdzono ponadto tendencję do wyższej aktywności $sPLA_2$ w przypadku większych stężeń izoprostanu 8-epi-$PGF_{2\alpha}$ III.

Wnioski: Aktywność $sPLA_2$ jest jednym z głównych czynników związanych z progresją chorób o tle zapalnym, w tym NAFLD. Obserwowana u mężczyzn pozytywna korelacja pomiędzy stężeniem cholesterolu całkowitego w osoczu i aktywnością $sPLA_2$, zgodnie z nową teorią „pojedynczego uderzenia", może spowodować progresję zapalenia wątroby.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is closely linked to accumulation of lipid droplets in hepatocytes [1]. A consequence of lipid accumulation in the liver is the autocatalytic process of lipid peroxidation, demonstrated by elevation of plasma lipid peroxidation markers – 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODEs) and isoprostane 8-epi-PGF$_{2\alpha}$ III. Both were significantly elevated in patients with nonalcoholic steatohepatitis (NASH) [2].

Phospholipases A$_2$ are enzymes releasing free fatty acids from cytoplasmic membranes and allowing their further transformation to HODEs, hydroxyeicosatetraenoic acids (HETEs) and eicosanoids. There are two main classes of phospholipases A$_2$, cytoplasmic (cPLA$_2$) and secretory phospholipase A$_2$ (sPLA$_2$), and both regulate physiological and pathological processes [3-5]. The sPLA$_2$ is expressed in the atherosclerotic arterial walls, highlighting the important role of that enzyme in the development of inflammation [3, 4, 6]. The role of sPLA$_2$ during progression of inflammation in NAFLD is practically unknown. However, lipoprotein-associated phospholipase A$_2$ was recently characterized as a novel inflammatory biomarker that is correlated with several components constituting the metabolic syndrome [7].

Aim

In this paper we look for plasma sPLA$_2$ activity in NAFLD patients. We also investigated the intensity of lipid peroxidation through measurement of plasma concentration of non-enzymatic oxidative stress markers: 9- and 13-HODEs, and isoprostane 8-epi-PGF$_{2\alpha}$ III.

Material and methods

Patients

Ninety-two consecutive patients with NAFLD were enrolled in the study as well as 20 healthy individuals. All analyzed subjects were Caucasians. The exclusion criterion was consumption of more than 20 g/day of ethanol. The subgroup of patients with clinically diagnosed NAFLD and permanently normal liver enzymes consisted of 30 individuals. All the patients tested negative for HBV infection (with HBs antigen and anti-HBc total antibodies) and anti-HCV antibodies. Gamma-globulins, immunoglobulins, and auto-antibodies were tested and imaging diagnostics was performed to exclude autoimmune hepatitis as well as primary biliary cirrhosis and primary sclerosing cholangitis. In a subgroup of 60 patients with biopsy-proven NAFLD, 12 patients (20%) had severe fibrosis of the liver defined as bridging fibrosis and cirrhosis (F3 and F4) and 24 (40%) had no fibrosis (F0) according to the Kleiner and Brunt classification [8]. Patients were fully informed as to the study objectives and benefits, and provided written consent prior to enrollment. The control group consisted of healthy individuals from general practitioners' registers. The study protocol complied with ethical standards laid down in the Declaration of Helsinki and was approved by the Committee on Human Research at the Pomeranian Medical University. Venous blood for lipid analyses was collected into tubes (with EDTA) after an overnight fast. After that, the plasma was obtained by centrifugation (1200 g × 10 min), and then frozen at –80°C until analyses.

Measurement of secretory phospholipase A$_2$ content in plasma

Before analysis samples were thawed slowly in an ice bath, vigorously vortexed and then equal amounts of samples were taken. The procedure was performed according to instructions of the manufacturer (Cayman, USA). Analysis was performed with En Vision 2104 Multilabel Reader (Perkin Elmer, USA).

Measurement of arachidonic and linoleic acid derivatives from plasma

Plasma 9- and 13-HODE were assessed with the HPLC method. First plasma (0.5 ml) was suspended with 1 ml of 100% acetonitrile (cooled to 4°C), then it was vortexed vigorously for 3 min. After that PGB2 (1 µg/ml) was added as an internal standard, and all was vortexed for the next 1 min. The mixture was cooled (10 min/–20°C), centrifuged (3200 g for 10 min, 4°C) and supernatant was taken. After that, the pH value of the samples was adjusted to 3.0 (with 1 mM HCl). The lipid fraction was extracted with solid-phase extraction SPE columns (Bakerbond Spe, RP-18, J. T Baker). Then derivatives were eluted by a mixture of methanol and ethyl acetate (1 : 1, v/v), carefully collected and evaporated to dryness in a nitrogen steam. Residue after evaporation was reconstituted in 200 µl methanol/water/acetic acid (60/40/0.1, v/v/v) and analyzed by HPLC. The Agilent 1200 chromatographic system consisted of a vacuum solvent degassing system, quaternary gradient pump, thermostated high-performance autosampler, thermostated column compartment and diode array detector (DAD). Chromatographic data were processed by Agilent Chemstation software as described in detail previously [9]. The total recovery for all sample extraction and processing steps was 46 ±8%.

Measurements of 8-epi-PGF$_{2\alpha}$ III concentration in plasma

Lipid fractions were extracted with a Folch mixture (chloroform : methanol 2 : 1, v/v) with butylated hydrox-

Table I. Main clinical differences between NAFLD males and females

Tabela I. *Różnice w parametrach klinicznych pomiędzy mężczyznami i kobietami chorującymi na NAFLD*

Parameter	Females (n = 33) (Mean ± SD)	Males (n = 59) (Mean ± SD)	Value of p
Age [years]	52.5 ±11.8	45.4 ±12.3	0.004
BMI [kg/m^2]	29.6 ±4.7	29.0 ±3.5	NS
Total cholesterol [mg/dl]	246.4 ±38	216.8 ±69.7	0.01
LDL cholesterol [mg/dl]	156.5 ±37	139 ±70.8	0.04
HODEs [ng/ml]	105.6 ±271.9	105.1 ±170.1	NS
8-epi-PGF$_{2\alpha}$ III [pg/ml]	96.5 ±113.2	128.6 ±175.5	NS

BMI – body mass index, HODE – hydroxyoctadecadienoic acid, 8-epi-PGF$_{2\alpha}$ III – isoprostane, NS – non-significant

Table III. Multivariate analysis of factors associated with sPLA$_2$ plasma concentration. The sPLA$_2$ and 8-epi-PGF$_{2\alpha}$ concentrations were transformed logarithmically

Tabela III. *Analiza wieloczynnikowa parametrów związanych ze stężeniem sPLA$_2$ w osoczu. Stężenia sPLA$_2$ i i 8-epi-PGF$_{2\alpha}$ przekształcono logarytmicznie*

Variable	β Coefficient	Value of p
Age	+0.124	0.44
Male gender	–0.316	0.043
BMI	–0.035	0.81
Serum total cholesterol	+0.428	0.0064
8-epi-PGF$_{2\alpha}$	–0.265	0.072

p = 0.010 for the whole multiple linear regression model

ytoluene (0.05%) as an antioxidant; the mixture was vortexed and centrifuged (3800 g × 10 min). The lower phase was then taken and evaporated under a stream of N_2. The sediment was saponified and hydrolyzed with 15% KOH and methanol (1 : 1, v/v). The mixture was vortexed and incubated at 37°C for 30 min. Then 1 mM of HCl was added and the mixture was applied to an SPE column (Bakerbond Spe, RP-18, J. T Baker). The 8-epi-PGF$_{2\alpha}$ III was eluted from the column by a mixture of ethyl acetate : methanol (1 : 1, v/v) and samples were evaporated under N_2. After that solvent was resuspended in dilution buffer, and assayed as described by the Cayman protocol. Analysis was performed with En Vision 2104 Multilabel Reader (Perkin Elmer, USA).

Statistical analysis

Statistica 7.1 software was used for the statistical analysis and all results are expressed as mean ± standard deviation. As the distribution in most cases deviated from normal (Shapiro-Wilk test), non-parametric Mann-Whitney test was used for comparisons between groups. A multiple linear regression model was used to find the independent predictors of sPLA$_2$ plasma concentration in NAFLD patients. Variables with distribution different from normal were transformed logarithmically before the multivariate analysis. Value of p below < 0.05 was considered significant.

Table II. Comparison between NAFLD males and females and sPLA$_2$ plasma concentration

Tabela II. *Porównanie stężenia sPLA$_2$ pomiędzy mężczyznami i kobietami chorującymi na NAFLD*

	NAFLD Females (n = 33) (Mean ± SD)	NAFLD males (n = 59) (Mean ± SD)	Value of p
sPLA$_2$ [pg/ml]	191 ±165	318 ±290	< 0.05

Table IV. Comparison of concentration of 9+13-HODE (measured by HPLC) and 8-epi-PGF$_{2\alpha}$ III (measured by ELISA kit) between groups of healthy individuals and NAFLD patients

Tabela IV. *Porównanie stężenia 9+13-HODE (mierzonego metodą HPLC) oraz 8-epi-PGF$_{2\alpha}$ III (mierzonego metodą ELISA) pomiędzy grupą osób zdrowych i pacjentów z NAFLD*

Parameter	Healthy (n = 20) (Mean ± SD)	NAFLD patients (n = 92) (Mean ± SD)	Value of p
sPLA$_2$ [pg/ml]	150 ±90	250 ±220	NS
9+13-HODE [ng/ml]	20.6 ±6.6	107.6 ±208.9	NS
8-epi-PGF$_{2\alpha}$ III [pg/ml]	15.66 ±10.2	116.4 ±154.5	0.000275

sPLA$_2$ – secretory phospholipase A$_2$, HODE – hydroxyoctadecadienoic acid, 8-epi-PGF$_{2\alpha}$ III – isoprostane

Results

Selected patients' data are summarized in Table I. The difference in content of sPLA$_2$ (among NAFLD individuals) between the genders nearly reached statistical significance (p = 0.054) (Table II). Additionally, among NAFLD patients multivariate analysis (adjusted for patient age, body mass index and gender as well as some biochemical data) showed that independent factors associated with higher sPLA$_2$ activity were higher total cholesterol concentration in plasma and (with borderline significance) female gender (Table III).

We did not find higher HODE concentration in plasma of the NAFLD subjects in comparison to healthy controls (Table IV), whereas concentration of isoprostane 8-epi-PGF$_{2\alpha}$ III was higher in NAFLD patients compared to healthy individuals (p = 0.00028).

Discussion

In this paper we have presented the results of evaluation of the inflammatory aspect of NAFLD pathogenesis.

We found an interesting correlation between quantity of sPLA$_2$ and male gender, and total plasma cholesterol concentration. In our previous study we found that male gender was associated with a lower level of severe fibrosis in the Polish NAFLD group [10]. It seems to be more interesting, although it is not certain, whether the higher activity of sPLA$_2$ observed in women was the result of more advanced age and dyslipidemia. On the other hand, it cannot be excluded that intensification of inflammatory processes (observed in women after menopause) may occur due to up-regulation of inflammatory enzymes, e.g. sPLA$_2$). The sPLA$_2$ is one of the main factors associated with progression of inflammatory disease (e.g. cardiovascular disease) and the serum level of lipoprotein-associated phospholipase A$_2$ was found to be elevated in NAFLD [7]. The enzyme plays a role by the activation of low-density lipoprotein (LDL) oxygenation and recruitment of macrophages to the atherosclerotic plaque [6]. Therefore a positive correlation between total cholesterol in plasma and activity of sPLA$_2$ is not surprising. The increase in cholesterol concentration is probably associated with the ongoing inflammatory process, in the development of which sPLA$_2$ is involved [6]. Based on the results of our study, we could support the thesis of Simonen *et al*. published recently [11]. Enhanced cholesterol synthesis leads in hepatocytes to excess free cholesterol, which is toxic to cells and could contribute to the pathogenesis of NAFLD and its progression to steatohepatitis [11]. Perhaps also lipids could be a bridge between inflammation in the liver and in the circulation. Fatty acids released by sPLA$_2$ are substrates for enzymes as well as free radical processes. Our study showed that the radical peroxidation product isoprostane 8-epi-PGF$_{2\alpha}$ III is increased in plasma of NAFLD patients.

Acknowledgments

This paper was supported by a grant from the State Committee for Scientific Research, 2006-2009, No. N 40209931/3037.

References

1. Federico A, D'Aiuto E, Borriello F, et al. Fat: a matter of distrubance for the immune system. World J of Gastroenterol 2010; 16: 4762-72.
2. Feldstein AE, Lopez R, Tamimi TA, et al. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. J Lipid Res 2010; 5: 3046-54.
3. Hurt-Camejo E, Camejo G. Potential involvement of type II phospholipase A2 in atherosclerosis. Atherosclerosis 1997; 132: 1-8.
4. Hurt-Camejo E, Camejo G, Peilot H, et al. Phospholipase A(2) in vascular disease. Circ Res 2001; 89: 298-304.
5. Han WK, Sapirstein A, Hung CC, et al. Cross-talk between cytosolic phospholipase A2 alpha (cPLA2 alpha) and secretory phospholipase A2 (sPLA2) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells: sPLA2 regulates cPLA2 alpha activity that is responsible for arachidonic acid release. J Biol Chem 2003; 278: 24153-63.
6. Hurt-Camejo E, Camejo G, Sartipy P. Phospholipase A2 and small, dense low-density lipoprotein. Curr Opin Lipidol 2000; 11: 465-71.
7. Colak Y, Senates E, Ozturk O, et al. Association of serum lipoprotein-associated phospholipase A2 level with nonalcoholic fatty liver disease. Metab Syndr Relat Disord 2012; 10: 103-9.
8. Kleiner DE, Brunt EM, Van Natta M, et al.; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005; 41: 1313-21.
9. Stachowska E, Dziedziejko V, Safranow K, et al. Effect of conjugated linoleic acids on the activity and mRNA expression of 5- and 15-lipoxygenases in human macrophages. J Agric Food Chem 2007; 55: 5335-2.
10. Raszeja-Wyszomirska J, Kurzawski G, Lawniczak M, et al. Nonalcoholic fatty liver disease and HFE gene mutations: a Polish study. World J Gastroenterol 2010; 16: 2531-6.
11. Simonen P, Kotronen A, Hallikainen M, et al. Cholesterol synthesis is increased and absorption decreased in non-alcoholic fatty liver disease independent of obesity. J Hepatol 2011; 54: 153-9.