Neutrophil effector responses are suppressed by secretory phospholipase A$_2$ modified HDL

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ABSTRACT

Secretory phospholipase A$_2$ (sPLA$_2$) generates bioactive lysophospholipids implicated in acute and chronic inflammation, but the pathophysiologic role of sPLA$_2$ is poorly understood. Given that high-density lipoprotein (HDL) is the major substrate for sPLA$_2$ in plasma, we investigated the effects of sPLA$_2$-mediated modification of HDL (sPLA$_2$-HDL) on neutrophil function, an essential arm of the innate immune response and atherosclerosis. Treatment of neutrophils with sPLA$_2$-HDL rapidly prevented agonist-induced neutrophil activation, including shape change, neutrophil extracellular trap formation, CD11b activation, adhesion under flow and migration of neutrophils. The cholesterol-mobilizing activity of sPLA$_2$-HDL was markedly increased when compared to native HDL, promoting a significant reduction of cholesterol-rich signaling microdomains integral to cellular signaling pathways. Moreover, sPLA$_2$-HDL effectively suppressed agonist-induced rise in intracellular Ca^{2+} levels. Native HDL showed no significant effects and removing lysophospholipids from sPLA$_2$-HDL abolished all anti-inflammatory activities.

Overall, our studies suggest that the increased cholesterol-mobilizing activity of sPLA$_2$-HDL and suppression of rise in intracellular Ca^{2+} levels are likely mechanism that counteracts agonist-induced activation of neutrophils. These counterintuitive findings imply that neutrophil trafficking and effector responses are altered by sPLA$_2$-HDL during inflammatory conditions.

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1. Introduction

Concentrations of some secretory phospholipase A$_2$ (sPLA$_2$) subspecies can increase hundred-folds in plasma during acute inflammation [1]. More than one third of the mammalian PLA$_2$ enzymes belong to the sPLA$_2$ family, which consists of low molecular mass, Ca^{2+}-dependent enzymes with a His–Asp catalytic dyad [2]. sPLA$_2$ enzymes, including groups IIA, III, V and X, catalyze the hydrolysis of glycerophospholipids to lysophospholipids and free fatty acids at the sn-2 position. Considerable evidence implicates a potential role for groups IIA and V sPLA$_2$ in cardiovascular disease and in patients with sepsis and septic shock, and increased plasma levels of sPLA$_2$ have been observed in acute inflammatory conditions, such as sepsis as well as in chronic inflammatory diseases [3–5].

Circulating levels of sPLA$_2$-IIA and increased sPLA$_2$ activities associate with cardiovascular risk in patients with sepsis and coronary artery disease [6]. Pathologic studies demonstrated the presence of sPLA$_2$ isoforms in atherosclerotic lesions and myocardial regions that have sustained ischemic injury [7]. Unexpectedly, the inhibition of sPLA$_2$ in patients with acute coronary syndromes resulted in an excess rate of myocardial infarction and stroke [8] and showed no overall survival benefit in patients with severe sepsis [9]. These studies clearly suggest that the pathophysiologic roles of sPLA$_2$ are poorly understood. Most sPLA$_2$ isoforms have the unique capacity to bind and hydrolyze lipoproteins in distinct and specific manners, thereby producing various lipid mediators and modifying the lipid particles. In the acute phase of the inflammatory response, sPLA$_2$ is mostly associated with high-density lipoproteins (HDL) [10], which are the major source of phospholipids in plasma [11] and are important players in the innate and adaptive immunity and cardiovascular disease [12–14]. sPLA$_2$ overexpression in apoA-I transgenic mice results in a dramatic shift of the HDL particle size toward smaller particles and virtually all plasma sPLA$_2$ is found in the HDL fraction [15]. Surprisingly, direct effects of sPLA$_2$ modified HDL on innate immune cells have not been assessed so far. Neutrophils are classically considered as short-lived phagocytes with the ability to release vast amounts of proteolytic enzymes and reactive oxygen species, both of which are important during bacterial infections. Thus, both

exuberant and/or diminished responses by the innate immune system may worsen clinical outcomes in severely ill patients with acute and chronic liver diseases [16], airway diseases [17] or acute coronary syndrome [18]. Neutrophils play key roles in sepsis [19] and atherosclerosis [20] and were shown to aggravate endothelial dysfunction, to activate macrophages, to promote foam cell formation and to contribute to weakening of the fibrous cap [20]. We therefore thought to assess whether sPLA$_2$-treated HDL modulates neutrophil function, an essential arm of the innate immune response.

2. Materials and Methods

2.1. Materials

All laboratory reagents were from Sigma (Vienna, Austria), unless otherwise specified. Interleukin 8 (IL-8) and intracellular adhesion molecule-1 (ICAM-1) were purchased from Peprotech (London, UK). Free fatty acids (FFA), lysophosphatidylcholines (LPC) and lysophosphatidylserine 16:0 were from Avanti Polar Lipids (Birmingham, AL, USA). FFAs were dissolved in ethanol and LPCs in chloroform/methanol and stored at −20 °C under argon atmosphere. Required amounts of LPC were dried under a stream of nitrogen and re-dissolved in PBS (pH 7.4). CellFix and FACS-Flow were from BD Bioscience (Vienna, Austria). Secretory phospholipase A$_2$ (sPLA$_2$) type III from bee venom and human recombinant secretory phospholipase A$_2$ type V were purchased from Cayman Europe (Tallin, Estonia). Varespladib was purchased from Eubio (Vienna, Austria). Anti-human CD11b-FITC and CD16-PE antibodies were obtained from Biozym-Biotech (Vienna, Austria). Fluo-3-AM and SYTOX green were from Life Technologies (Vienna, Austria). Fixative solution was prepared by adding 9 ml distilled water and 30 ml FACS-Flow to 1 ml CellFix.

2.2. Isolation of HDL

HDL was isolated by density gradient ultracentrifugation as described [21–23]. Plasma density was adjusted with potassium bromide (Sigma, Vienna, Austria) to 1.24 g/ml, and a two-step density gradient was generated in centrifuge tubes (16 × 76 mm, Beckman) by layering the density-adjusted plasma (1.24 g/ml) underneath a NaCl-density solution (1.006 g/ml). Tubes were sealed and centrifuged at 90,000 rpm for 4 h in a 90Ti fixed angle rotor (Beckman Instruments, Krefeld, Germany). After centrifugation, the HDL-containing band was collected and desalted via PD10 columns (GE Healthcare, Vienna, Austria) and immediately used for experiments.

2.3. sPLA$_2$ treatment of HDL

HDL was incubated in the presence of 200 ng/ml sPLA$_2$ type III from bee venom or 400 ng/ml human recombinant type V sPLA$_2$ in PBS containing Ca^{2+} and Mg^{2+}, overnight at 37 °C, in order to hydrolyse HDL-associated phospholipids.

2.4. Lysophosphatidylcholine (LPC), free fatty acid (FFA) and lysophosphatidylserine enrichment/depletion of HDL

In order to generate LPC-, FFA-, or lysophosphatidylserine-enriched HDL, 1 mg/ml HDL was incubated with 0.6 mmol/L 16:0, 18:1, 18:2 or 20:4 FFA, with 0.6 mmol/L 16:0, 18:1, 18:2 or 20:4 LPC or with 0.6 mmol/L lysophosphatidylserine 16:0 for 2 h at 37 °C. Unbound LPCs and FFAs were removed by gel filtration, and HDL-associated LPC and FFA contents were determined as described below. In some experiments, sPLA$_2$-treated HDL was incubated in the presence or absence of 50 mg/ml albumin (1 h, 37 °C) and sPLA$_2$-HDL was re-isolated by density gradient ultracentrifugation in order to remove LPCs and FFAs. LPC and FFA contents were assessed as described below.

2.5. Determination of FFAs and LPCs

HDL-associated lipids were extracted according to Bligh and Dyer [24] and dried under a stream of nitrogen. Dried lipid extracts were resuspended in 200 μl CHCl$_3$/MeOH (1:1, v/v) containing 1 pmol/μl of LPC 17:1 serving as internal standard. Chromatographic separation of lipids was performed by an Accela HPLC (Thermo Scientific) on a Thermo Hypersil GOLD C18, 100 × 1 mm, 1.9 μm column. Solvent A was a water solution of 1% ammonium acetate (v/v) and 0.1% formic acid (v/v), and solvent B was acetonitrile/2-propanol (5:2, v/v) supplemented with 1% ammonium acetate (v/v) and 0.1% formic acid (v/v), respectively. The gradient was run from 35% to 70% B for 4 min, then to 100% B in additional 16 min with subsequent hold at 100% for 10 min. The flow rate was 250 μl/min. Phospholipid species were determined by a TSQ Quantum ultra (Thermo Scientific) triple quadrupole instrument in positive ESI mode. The spray voltage was set to 4500 V and capillary voltage to 35 V. The LPC species were detected in a precursor ion scan on m/z 184 at 34 eV. Peak areas were calculated by QuanBrowser for all lipid species and the calculated peak areas for each species were expressed as a % of internal standard. Results are expressed as nmol/mg HDL protein. FFA content was assessed using a non-esterified fatty acids kit (Diasys, Holzheim, Germany). In some experiments, the Azwell LPC Assay Kit (Hölzl Diagnostika) was used to assess the LPC content of sPLA$_2$-HDL.

2.6. Neutrophil isolation

Human polymorphonuclear leukocytes (PMNL) were isolated as previously described [25,26], from peripheral blood of healthy volunteers according to a protocol approved by the Ethics Committee of the Medical University of Graz. Prior to blood sampling from healthy volunteers, all donors signed an informed consent form. Platelet-rich plasma was removed by centrifugation of citrated whole blood. Erythrocytes were removed by dextran sedimentation. PMNL were isolated by Histopaque gradient centrifugation. Any erythrocyte contamination of the PMNL pellet was removed by hypotonic shock lysis. The purity and viability of neutrophil preparation was greater than 95%. All functional assays of neutrophils were performed in assay buffer (PBS with Ca^{2+} and Mg^{2+}, HEPES 10 mmol/L, glucose 10 mmol/L, bovine serum albumin 0.1%, pH 7.4).

2.7. Neutrophil shape change assay

Neutrophil shape change was measured as previously described [27, 28]. Isolated PMNL were resuspended in assay buffer, and aliquots of cells (about 3 × 10^5 cells per sample) were preincubated with HDL samples and then stimulated in 37 °C shaking water bath with interleukin-8 (IL-8), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or complement component 5a (C5a) for 4 min, with lipopolysaccharide (LPS) in the presence of 2% serum for 90 min or with *Escherichia coli* bacteria for 60 min at a final volume of 100 μl. Cells were transferred to ice, and 150 μl of ice-cold fixative solution was added to terminate the reaction and maintain the change in cell shape until analysis. The samples were then analyzed on a FACScalibur flow cytometer (BD Biosciences). Eosinophils were distinguished from neutrophils according to granularity (side scatter) and by their autofluorescence in the FL-2 channel. Shape change was determined as the increase in the forward scatter property of the cell compared with vehicle stimulation.

2.8. CD11b activation

Isolated PMNL were preincubated with HDL samples and stimulated with IL-8 (3 nmol/L), fMLP (5 nmol/L) or C5a (30 nmol/L) for 4 min at 37 °C in shaking water bath in the presence of FITC-conjugated Ab to the active epitope of CD11b. Cells were fixed and then analyzed by flow cytometry [29].