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Analysis of sphingolipid and prostaglandin synthesis during zymosan-induced inflammation

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ABSTRACT

Sphingosine-1-phosphate (S1P) is generated through phosphorylation of sphingosine by two sphingosine kinases (SPHK-1 and -2). As extra- and intracellular messenger S1P fulfils multiple roles in inflammation such as mediating proinflammatory inputs or acting as chemoattractant. In addition, S1P induces cyclooxygenase-2 (COX-2) expression and the synthesis of proinflammatory prostanoids in several cell types. Here, we analysed in vivo the regulation of S1P level as well as potential interactions between S1P and COX-dependent prostaglandin synthesis during zymosan-induced inflammation. S1P and prostanoid levels were determined in the blood and at the site of inflammation under basal conditions and during zymosan-induced inflammation using wild type and SPHK-1 and -2 knockout mice. We found that alterations in S1P levels did not correlate with changes in plasma- or tissue-concentrations of the prostanoids as well as COX-2 expression. In the inflamed tissue S1P and prostanoid concentrations were reciprocally regulated. Prostaglandin levels increased over 6 h, while S1P and sphingosine level decreased during the same time, which makes an induction of prostanoid synthesis by S1P in zymosan-induced inflammation unlikely. Additionally, despite altered S1P levels wild type and SPHK knockout mice showed similar behavioural nociceptive responses and oedema sizes suggesting minor functions of S1P in this inflammatory model.

1. Introduction

Lipids are important mediators of a multitude of biological functions. Especially in inflammatory settings lipids, such as prostaglandins (PG), have been identified as essential mediators of numerous pro- and anti-inflammatory processes [1,2]. For example, cyclooxygenase-2 (COX-2) and the functionally coupled microsomal PGE_2 synthase-1 (mPGES-1) catalyse the generation of PGE_2 [3,4] whose well known pro-inflammatory properties are the reason for the clinical use of the non-steroidal anti-inflammatory drugs (NSAIDs) [1,5–8]. More recently also sphingolipids have been implicated in modulating inflammatory processes involved in cardiovascular diseases, tumourigenesis, transplantation and pain [9–12].

S1P is synthesized by phosphorylation of sphingosine by two sphingosine kinases (SPHK-1 and -2) which exhibit both overlapping and distinct physiological functions [13]. S1P is produced in a wide variety of cell types and can mediate its actions either through the activation of a family of five G-protein coupled receptors (S1P1–5) or by acting as an intracellular second messenger [14–16]. Several studies suggest an integral role for S1P in mediating inflammatory responses including functions as chemoattractant, in lymphocyte differentiation and – trafficking as well as in Toll-like receptor (TLR) – tumour necrosis factor α (TNFα)- and protease-activated receptor-1-(PAR1-) signalling [17]. Also, SPHK-1 has been reported to regulate COX-2 expression in various cell types [18–22] and down-regulation of SPHK-1 prevented cytokines from inducing COX-2 expression [21,22].

In vivo, extracellular S1P was demonstrated to be an essential factor allowing lymphocyte egress from lymphnodes and acting as chemoattractant for various immune cells including leucocytes [17,23]. Confusingly, for macrophages it has been shown that during thioglycollate-induced peritonitis the number of peritoneal macrophages was reduced in S1P3-deficient mice [24] but increased in S1P2-deficient mice [25]. Furthermore, neutrophil functions were not altered in SPHK-1 or -2 knockout mice and SPHKs appear not to be essential for neutrophil recruitment and neutrophil functions [26].

Abbreviations: BL, baseline; COX-1/2, cyclooxygenase-1/2; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PG, prostaglandin; PWL, paw withdrawal latency; S1P, sphingosine-1-phosphate; SPHK, sphingosine kinase; Tx, thromboxane.

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In the present study, we aimed to (1) define the S1P and prostanoid concentrations in the blood as well as paw tissue, (2) determine their regulation in a well described inflammatory model, (3) study their regulation in SPHK-1 and -2 deficient mice, and (4) investigate potential interaction between both groups of lipid mediators during inflammation. Therefore, COX-2 expression, prostanoid and S1P levels as well as oedema size and behavioural responses were analysed in wild type, SPHK-1- and SPHK-2-deficient mice.

2. Materials and methods

2.1. Animals

SPHK-1 and SPHK-2-deficient mice were kindly provided by Dr. Andreas Billich, Novartis, Switzerland [27]. Knockout mice were compared with strain-, age-, and sex matched controls. In all experiments the Ethics guidelines of the Public Health Services (PHS) for investigations in conscious animals were obeyed and the procedures were approved by the local Ethics Committee.

2.2. Materials

Diclofenac sodium salt was purchased from Sigma–Aldrich (Seelze, Germany).

2.3. Western blot

Tissue or cell lysates (20 μg protein) were separated on a 15% SDS polyacrylamide gel. After blotting, SPHK-2 (Santa Cruz, CA, USA) or COX-2 (Acris, Herford, Germany) were detected with a polyclonal antibody. Anti-HSP-90 (BD Biosciences, Heidelberg, Germany) antibody was used to control for equal loading.

2.4. Behavioral tests

20 μl of zymosan (3 mg/ml) was injected in one hind paw. Zymosan-induced thermal hyperalgesia was determined as described previously [28]. The observer was unaware of the treatments and genotypes in all tests.

2.5. Paw oedema size

The volume of the paw oedema was determined at the indicated times after injection of 20 μl zymosan (3 mg/ml) in one hind paw. A plethysmometer (IITC Life Science, CA, USA) was used to measure the oedema size by immersion of the animal's paw, calibrated in millilitre.

2.6. Isolation of blood cells

Mice blood was collected by cardiac puncture and citrate was added to a final concentration of 10%. White blood cells (WBCs) and red blood cells (RBCs) were sedimented by centrifugation at 150 × *g* for 10 min at room temperature (RT). The platelet containing solution was centrifuged at 1300 × *g* for 5 min at RT to generate platelet poor plasma. WBCs and RBCs were resuspended in 2 ml erythrocyte lysing solution (135 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM Na-EDTA, pH 7.2), gently mixed and incubated at room temperature for 5 min. WBCs were sedimented by centrifugation at 300 × *g* for 10 min. Cells were resuspended in 2 ml erythrocyte lysing solution, incubated at room temperature for 5 min and centrifuged at 300 × *g* for 20 min. The WBC pellet was resuspended and homogenized in Hanks' Balanced Salt Solution (HBSS) containing $CaCl_2$ and $MgCl_2$ (Invitrogen, Carlsbad, CA) and 10% citrate.

2.7. Blood cell count

Blood was collected by puncture of the heart, citrate was added to a final concentration of 10% and blood cells were counted using a Cell Dyne cytometer (Abbott Diagnostics, Wiesbaden, Germany) according to the manufacturer's instruction.

2.8. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS)

Whole blood, plasma and tissue of the paw oedema were directly frozen with liquid nitrogen. 200 μl PBS was added to the tissue of one paw and homogenized with a potter. 100 μl of the tissue homogenate was used for lipid extraction.

2.9. Sphingolipids

Sphingolipid concentrations were determined after addition of internal standards and extraction in chloroform/MeOH (2× 600 μl, 6.25 ml MeOH + 1 ml HCl, 50 ml chloroform). HPLC analysis was done under gradient conditions using a Luna C18-column (150 cm × 2 mm, Phenomenex, Aschaffenburg, Germany). MS/MS analyses were performed on a 4000 Q TRAP triple quadrupole mass spectrometer with a Turbo V source (Applied Biosystems, Darmstadt, Germany) as described previously [29]. Concentrations of the calibration standards, quality controls and samples were evaluated by Analyst software 1.4 (Applied Biosystems, Darmstadt, Germany). The coefficient of correlation for all measured sequences was at least 0.99. Variations in accuracy and intra-day and inter-day precision ($n = 6$ for each concentration, respectively) were <15% over the range of calibration.

2.10. Prostaglandins

The following eicosanoids and their stable metabolites were monitored by LC–MS/MS: $PGF_{2\alpha}$, PGE_2, PGD_2, TxB_2 (stable metabolite of TxA_2) and 6-keto-$PGF_{1\alpha}$. 100 μl of the homogenate was mixed with 50 μl water, 20 μl methanol and 20 μl internal standard solution (25 ng/ml of [2H4]-PGE_2, [2H4]-PGD_2 and [2H4]-TxB_2 and 10 ng/ml of [2H4]-$PGF_{2\alpha}$ and [2H4]-6-keto-$PGF_{1\alpha}$ in methanol) and extracted twice with 800 μl ethyl acetate. The organic phase was removed at a temperature of 45 °C under a gentle stream of nitrogen. The residues were reconstituted with 50 μl of acetonitrile/water/formic acid (20:80:0.0025, v/v, pH 4.0), and injected into the LC–MS/MS system [30].

2.11. Statistics

Results were analysed using one-way analysis of variance (ANOVA) followed by Student's *t*-test and Bonferroni correction where applicable. All results were tested for Gaussian distribution and were tested in case of non-Gaussian distribution with a non-parametric test (Mann–Whitney). Significance was accepted at $P < 0.05$.

3. Results

First, we investigated potential haematological changes in SPHK knockout mice in comparison to wild type animals. We found that the cell count of neutrophils, lymphocytes and monocytes was unaltered in the peripheral blood (Fig. 1A–C). While erythrocyte counts in wild type and SPHK knockout mice were comparable (Fig. 1D), the platelet count was decreased by about 30% in SPHK-2 deficient mice (Fig. 1E). Notably, platelets are besides erythrocytes and endothelial cells one of the sources of S1P in the blood [31–34].