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Regulation of endothelial nitric oxide synthase activation in endothelial cells by $S1P_1$ and $S1P_3$

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ABSTRACT

Endothelial nitric oxide synthase (eNOS) plays a crucial role in vascular homeostasis. Lysophospholipid interaction with sphingosine 1-phosphat (S1P) receptors results in eNOS activation in different cells. In endothelial cells, eNOS activation via $S1P_1$ or $S1P_3$ was shown controversially. The aim of this study is to investigate the meaning of both S1P receptors for eNOS activation in human endothelial cells. Therefore, several $S1P_1$ and $S1P_3$ agonists in combination with antagonists and specific RNAi approach were used. eNOS activation was measured in human umbilical vein endothelial cells (HUVEC) via DAF2-DA-based fluorescence microscopy. For investigation of the signaling pathway, agonists/antagonist studies, RNAi approach, Luminex™ multiplex, and Western Blot were used. In HUVEC, both the $S1P_1$ agonist AUY954 as well as the $S1P_{1,3}$ agonist FTY720P induced eNOS activation in a time- and dose-dependent manner. Other $S1P_1$ agonists activated eNOS to a lesser extent. The AUY954-induced eNOS activation was blocked by the $S1P_1$ antagonist W146, the combination of W146 and the $S1P_3$ antagonist CAY10444 and the $S1P_{1,3}$ antagonist VPC23019, but not by CAY10444 indicating the meaning of $S1P_1$ for the AUY954-induced eNOS activation. The FTY720P-induced eNOS activation was blocked only by the combination of W146 and CAY10444 and the combined $S1P_{1,3}$ antagonist VPC23019, but not by W146 or CAY10444 indicating the importance of both $S1P_1$ and $S1P_3$ for FTY720-induced eNOS activation. These results were confirmed using specific siRNA against $S1P_1$ and $S1P_3$. The $S1P_{1,3}$ activation results in Akt phosphorylation and subsequent activation of eNOS via phosphorylation at serine^{1177} and dephosphorylation at threonine^{495}. Beside former investigations with rather unspecific S1P receptor activation these data show potent selective $S1P_1$ activation by using AUY954 and with selective S1P receptor inhibition evidence was provided that both $S1P_1$ and $S1P_3$ lead to downstream activation of eNOS in HUVEC in the same experimental setting. Inhibition or knockdown of one of these receptor subtypes did not abolish the eNOS activation and subsequent NO production.

1. Introduction

The endothelium has pleiotropic functions and is involved in the regulation and homeostasis of the vasculature [1]. Here, nitric oxide (NO) generated by the endothelial NO synthase (eNOS) plays a major role [2]. Furthermore, eNOS modulates platelet aggregation, vascular remodelling and angiogenic processes [3]. A variety of physiological and pathophysiological stimuli modulate NO production via eNOS [4]. Of these, lysophospholipids, especially such as sphingosine-1-phosphate (S1P), activate eNOS in a G-protein coupled receptor (GPCR)-dependent manner [4–6]. S1P is involved in many physiological as well as in pathophysiological processes of arterial vascular diseases [7]. S1P acts as intracellular second messenger or activates GPCR-dependent S1P receptors on the cell surface. In the cardiovascular system, three of five known S1P receptors, $S1P_{1,2,3}$, are the major subtypes mediating multiple vascular signaling processes [7,8]. The role of high density lipoprotein (HDL)-associated S1P as well as non-HDL-bound-S1P in eNOS-mediated vasodilatation has been verified in several experimental settings [9,10]. The intracellular signaling pathway includes the Akt-mediated eNOS phosphorylation [6,10–13]. Several studies using different experimental settings in different cells have shown that either $S1P_1$ or $S1P_3$ is involved in eNOS signaling [10,13–16]. However, due to so far unselective receptor activation it remains

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unclear whether $S1P_1$ or $S1P_3$ alone or both are necessary for eNOS-mediated NO production.

During the last few years, selective agonists and antagonists for S1P receptors have been established. In the current experiments, selective agonists for $S1P_1$ (AUY954) and $S1P_{1,3}$ (FTY720P) and selective antagonists for $S1P_1$ (W146), $S1P_3$ (CAY10444), and $S1P_{1,3}$ (VPC23019) were used.

In summary, selective descrimination of S1P receptor subtypes provide evidence that both $S1P_1$ as well as $S1P_3$ activation result in NO production via Akt-mediated eNOS stimulation in human umbilical vein endothelial cell (HUVEC). Inhibition or knockdown of only one of these receptor subtypes did not abolish the NO production in HUVEC.

2. Materials and methods

2.1. Materials

AUY954 and FTY720P were a kind gift from Novartis Pharma AG (Basel, Switzerland). Cay10444, KRP203P and SEW2871 were obtained from Cayman Chemicals (Ann Arbor, USA). Akt inhibitor IV was purchased from Calbiochem (Darmstadt, Germany). VPC24191, VPC23019 and W146 were obtained from Avanti Polar Lipids (Alabaster, USA).

2.2. Cell culture

HUVEC of pooled donors were obtained from PromoCell (Heidelberg, Germany) and cultured at 37 °C, 95% humified atmosphere and 5% CO_2 in Endothelial Cell Basal medium including supplement (PromoCell, Heidelberg, Germany). The experiments were conducted with cell passages 3–8 and experiments were performed as indicated times in individual experiments.

2.3. Detection of NO via fluorescence microscopy

HUVEC were seeded on μ-dishes (Ibidi, Martinsried, Germany) and were serum-starved (0.5% supplement) for 24 h. In case inhibitors were used, cells were pre-incubated for 30 min prior to stimulation. Cells were stimulated as indicated. Staining with DAF2-DA (5 μmol/L; Invitrogen, Karlsbad, Germany) was carried out in the dark for the last 20 min of stimulation procedure. Afterwards, cells were washed with phosphate buffered saline (PBS) and fixed with glutaraldehyde (2%) for 3 min at 4 °C. Fluorescence intensity was detected in defined exposure time settings for all samples using an AxioVert 200M (Zeiss, Jena, Germany; excitation: 470 nm, emission: 515 nm) and quantified using the AxioVisio Software (Zeiss, Jena, Germany). Three images out of one stimulation setting were analyzed and fluorescence values per area and image were counted. Subsequently, the mean ± SEM of independent experimental settings were calculated and normalized to controls.

2.4. siRNA transfection

HUVEC were serum-starved (0.5% supplement) for 48 h pre-transfection. Cells were trypsinised then counted and transfected in suspension. 0.5×10^6 cells were transfected with predesigned silencer select siRNA (Applied Biosystems, Darmstadt, Germany) and negative control siRNA with similar GC level (Invitrogen, Karlsbad, Germany) using the Amaxa nucleofector system with the HUVEC nucleofector kit solution (Lonza, Verviers, Belgium). Mock transfected cells were used as a control. Transfected HUVEC were seeded on μ-dishes (Ibidi, Martinsried, Germany), were cultured for 48 h and afterwards the cells were serum-starved (0.5% supplement) for 24 h. Cells were stimulated as indicated 72 h post-transfection. Knockdown efficacy was verified using real-time PCR.

2.5. Western Blot

HUVEC were serum-starved (0.5% supplement) for 24 h prior to stimulation. In case antagonists were used, they were added to the cells 30 min prior to stimulation. Cells were stimulated as indicated. After washing with PBS, cells were harvested in cell lysis buffer (Invitrogen, Karlsbad, Germany) containing a protease inhibitor mix (Roche, Grenzach, Germany) and PMSF (1 mmol/L, Sigma-Aldrich, Steinheim, Germany). Protein concentrations were determined using a BCA™ assay kit (Pierce, Rockford, USA). 20 μg of protein were loaded on a 12% SDS-PAGE (Pierce, Rockford, USA), separated by electrophoresis and transferred to a nitrocellulose membrane using the iBlot system (Invitrogen, Karlsbad, Germany). Membranes were incubated with a specific mouse anti-eNOS (1:1000; BD, Heidelberg, Germany) or rabbit anti-phospho-eNOS antibody (Thr^{495}: 1:500 and Ser^{1177}: 1:1000) (Cell Signaling, Danvers, USA) overnight at 4 °C. After washing with TBST, the membranes were incubated for 2 h at room temperature with HRP-conjugated anti-mouse (KPL, Gaithersburg, USA) and anti-rabbit antibody (KPL, Gaithersburg, USA) respectively, washed again with TBST and incubated with Super Signal Dura West substrate (Pierce, Rockford, USA). Signal detection was performed by using the ChemiSmart imaging system (Vilber Lourmat, Torcy, France). For quantification of band intensities, the Bio1D imaging software (Vilber Lourmat, Torcy, France) was used.

2.6. Phosphoprotein detection

HUVEC were cultivated in low-serum medium (0.5% supplemental growth factors) for 48 h. Cells were stimulated as indicated. Afterwards, the cells were harvested in ice-cold cell lysis buffer including protease inhibitors (Biorad, Muenchen, Germany) and were centrifuged for 20 min at 4 °C, 13,000 rpm. Supernatant was diluted with assay buffer (Biorad, Muenchen, Germany). Determination of phosphorylated Akt protein and total Akt protein was performed by Luminex™ technology using the phospho-protein detection assay (Biorad, Muenchen, Germany). Protein amount of the cell lysates were determined with BCA™ assay kit (Pierce, Rockford, USA). Ratio phospho/total was normalized to protein content of the lysates.

2.7. Statistical analysis

Data are presented as means ± SEM from at least three separate experiments or as results representative of at least three repetitions, unless otherwise indicated. Calculations were done with GraphPad Prism software (Version 5.03, GraphPad Software, Inc., La Jolla, USA). Comparisons between the groups were performed with two-tailed Mann-Whitney *U* test. A p value ≤ 0.05 was considered significant.

3. Results

3.1. eNOS activation and subsequent NO production induced by $S1P_1$ and $S1P_3$ agonists

Prior studies showed that activation of $S1P_{1,3}$ leads to an induction of eNOS [9]. In the current study HUVEC were used for all experiments. HUVEC express $S1P_{1,3}$ (data not shown). In the present study, the $S1P_{1,3}$ agonist VPC24191, the $S1P_{1,3,4,5}$ agonist FTY720P, and the selective $S1P_1$ agonists AUY954 and KRP203P activated eNOS in a time-dependent (0–90 min) manner. The $S1P_1$