Original Paper

PI3K β Plays a Key Role in Apolipoprotein A-I-Induced Endothelial Cell Proliferation Through Activation of the Ecto-F₁-ATPase/P2Y₁ Receptors

Audrey Castaing-Berthou^{a,b} Nicole Malet^{a,b} Claudia Radojkovic^c
Cendrine Cabou^{a,b} Stéphanie Gayral^{a,b} Laurent Olivier Martinez^{a,b}
Muriel Laffargue^{a,b}

^aInstitut National de la Santé et de la Recherche Médicale (INSERM), UMR 1048, Institute of Metabolic and Cardiovascular Diseases, ^bUniversity of Toulouse, UMR1048, Paul Sabatier University, Toulouse, France; ^cDepartment of Clinical Biochemistry and Immunology, Faculty of Pharmacy, University of Concepción, Concepción, Chile

Key Words

ATP synthase • HDL • Apolipoprotein A-I • Phosphoinositide 3-kinase • Purinergic signaling • Endothelial cell

Abstract

Background/Aims: High-density lipoproteins (HDL) exert multiple cardioprotective functions on the arterial wall, including the promotion of endothelial cell survival and proliferation. Among mechanism contributing to endothelial protection, it has been reported that apolipoprotein A-I (apoA-I), the major protein in HDL, binds and activates the endothelial ecto-F₁-ATPase receptor. This generates extracellular ADP, which in turn promotes endothelial cell survival. In this study, we aimed to further investigate the signaling pathway involved downstream of apoA-I-induced ecto-F₁-ATPase activation.

Methods: In human umbilical vein endothelial cells (HUVECs), pharmacological and gene silencing approaches were used to study pathways involved downstream ecto-F₁-ATPase activation by apoA-I. **Results:** ApoA-I and HDL both induced Akt phosphorylation. F₁-ATPase inhibitors such as inhibitory factor 1 and oligomycin completely blocked apoA-I-induced Akt phosphorylation and significantly blocked HDL-induced phosphorylation, indicating that this signaling pathway is dependent on ecto-F₁-ATPase activation by apoA-I. Further, we were able to specify roles for the P2Y₁-ADP receptor and the PI3K β isoform in this pathway since pharmacological inhibition and silencing of these proteins dramatically inhibited apoA-I-induced Akt phosphorylation and cell proliferation. **Conclusion:** Altogether, these data highlight a key role of the P2Y₁/PI3K β axis in endothelial cell proliferation downstream of ecto-F₁-ATPase activation by apoA-I. Pharmacological targeting of this pathway could represent a promising approach to enhance vascular endothelial protection.

© 2017 The Author(s)
Published by S. Karger AG, Basel

M. Laffargue and L.O. Martinez contributed equally to this work.

L.O. Martinez and M. Laffargue

INSERM UMR1048, Bât. L3, Hôpital Rangueil, BP 84225, 31432 Toulouse cedex 04 (France)
Tel. +33 (0)5 31 22 41 47, E-Mail laurent.martinez@inserm.fr/muriel.laffargue@inserm.fr

Introduction

Atherosclerosis is a multifactorial and multistep disease that causes coronary artery diseases, peripheral vascular diseases and stroke. Its incidence is increasing worldwide and is the major cause of death due to cardiovascular events. The earliest stage in the development of atherosclerosis is characterized by altered normal endothelial homeostatic functions, which promote the retention and oxidation of apoB-lipoproteins in the sub-endothelial space and are associated with an inflammatory response. This leads to *i*) the differentiation of intima-infiltrated monocytes into macrophages, causing them to accumulate cholesterol, become foam cells and eventually form a lipid core, and *ii*) the phenotypic modulation of smooth muscle cells, switching them from a contractile to a synthetic phenotype to promote neointimal hyperplasia. The treatment of symptomatic atherosclerosis typically involves an angioplasty with stent placement but this procedure further damages the vascular endothelium and frequently triggers thrombosis and restenosis [1]. Thus, endothelial protection and repair remain key processes to consider when developing strategies to help prevent atherosclerosis and its complications [2].

Large prospective population studies have provided evidence that low levels of high-density lipoprotein cholesterol (HDL-C) are a major independent cardiovascular risk factor [3]. Accordingly, pre-clinical and translational studies have also revealed that HDL particles (HDL) display multiple anti-atherogenic properties [4, 5], with the best known property being their ability to promote cellular cholesterol efflux and the transport of cholesterol back to the liver for excretion. In addition, HDL are known to exert beneficial effects on endothelial cells, including the stimulation of nitric oxide (NO) production, the inhibition of the apoptotic action of stimuli such as TNF- α and oxidized LDL, and the promotion of cell proliferation and migration [6, 7]. However, recent results from genetic or clinical research have cast considerable doubt on the relevance of increasing HDL-C concentration *per se* [8]. First, Mendelian randomization studies of common genetic variants for HDL-C are inconsistent in their support for a causal relationship between HDL-C concentration and cardiovascular diseases [9, 10]. Second, in clinical trials, HDL-C raising drugs, such as nicotinic acid and CETP inhibitors, have failed to reduce cardiovascular events any more than statins administered alone [8, 11]. One argument to explain these discrepancies is that assessment solely on HDL-C might not predict the outcome of pharmacological intervention. Indeed, HDL-C concentration is a static measurement and does not reflect the dynamics or the functionality of the HDL particles, which differ according to the individual's metabolic status [12]. Thus, understanding the molecular mechanisms that underlie the biological activities of HDL will allow for more strategic approaches in the development of HDL-based therapeutics.

Interestingly, both the protein and lipid moieties of HDL contribute to their beneficial effects on endothelial cells. Early *in vitro* studies reported that both HDL and apolipoprotein A-I (apoA-I), the main HDL apolipoprotein, stimulate endothelial cell proliferation and migration and inhibit apoptosis [13–15]. Consistent with this, carotid artery re-endothelialization following perivascular artery injury was shown to be diminished in apoA-I-null mice, with normal re-endothelialization restored by apoA-I reconstitution [16]. To date, three receptors that contribute to HDL-mediated cell survival signaling pathways have been identified on endothelial cells. First, sphingosine 1-phosphate (S1P) receptors interact with HDL-associated S1P and exert a mitogenic effect through activation of a phosphoinositide 3-kinase (PI3K)/Akt-dependent pathway [17]. HDL also bind to scavenger receptor class B member 1 (SR-BI) to accelerate re-endothelialization through activation of the Src kinases, PI3K and p44/42 mitogen-activated protein kinases (MAPK) [16]. Finally, apoA-I binds and activates the ecto-F₁-ATPase receptor on the surface of endothelial cells and endothelial progenitor cells (EPC), to promote cell proliferation [18, 19]. The effects of apoA-I on ecto-F₁-ATPase activity are strictly limited to this receptor since they were observed following the inhibition of other HDL/apoA-I receptors, such as SR-BI and ABCA1, but were reduced following treatment with inhibitory factor 1 (IF1-H49K), a specific F₁-

ATPase inhibitor. Interestingly, the effects of apoA-I-induced ecto-F₁-ATPase activity have been shown to depend on the production of extracellular ADP [18, 20], suggesting that an ADP-sensitive P2Y receptor might be involved, as has been previously shown in hepatocytes [21–24]. However, neither the P2Y receptor nor the intracellular signaling components responsible for endothelial cell proliferation downstream of ecto-F₁-ATPase activation have been characterized.

In this study, we first sought to gain a better understanding of the signaling pathways downstream of ecto-F₁-ATPase activation by apoA-I. Due to the central role of the PI3K/Akt pathway in HDL-mediated endothelial protection, we investigated whether this pathway was also involved downstream of apoA-I-induced ecto-F₁-ATPase activation, and thereby influences endothelial cell proliferation. Furthermore, we investigated the involvement of different P2Y ADP receptors and PI3K isoforms in this process.

Materials and Methods

Materials

Cell culture reagents were purchased from Lonza (Walkersville, USA). Rabbit monoclonal antibodies against phosphorylated Akt (anti-pAkt Ser⁴⁷³ and anti-pAkt Thr³⁰⁸), rabbit polyclonal antibodies against Akt (anti-Akt), HRP-conjugated anti-rabbit IgG antibodies and oligomycin were from Cell Signaling Technologies (Danvers, MA, USA). Rabbit polyclonal antibodies against the p110 β isoform of PI3K (anti-PI3K β) were from Santa Cruz Biotechnology (Dallas, Texas, USA). Sphingosine-1-phosphate (S1P) and MRS2179 (a P2Y₁ antagonist) were from Sigma Aldrich (Saint Quentin Fallavier, France). PI3K inhibitors (AS-252424, wortmannin and TGX-221) were from Selleckchem (Euromedex, France). PI3K β siRNA (Hs-PIK3CB_5, NM_006219), P2Y₁ siRNA (Hs_P2Y1_5_HP, NM_002563) and control siRNA (Allstars Neg Control siRNA) were from Qiagen (France). The enhanced chemiluminescence (ECL) system was from Bio-Rad (Roane, France). HDL₃, referred to as HDL throughout the manuscript, and apoA-I, were isolated from the plasma of normolipidemic healthy human donors as previously described [25]. The plasmid encoding the mutated IF₁ protein (with histidine 49 switched to lysine, referred to as IF1-H49K), which is active at all pH values, was kindly provided by J. Walker (Cambridge, UK), and protein production was carried out as previously described [26].

Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in EBM-2 medium containing 2% (v/v) fetal bovine serum (FBS) and growth factors (EGM-2 Bullet Kit, Lonza). Cells were seeded in collagen I-coated dishes (BD biocoat, Becton Dickinson) at a density of 5000 cells/cm² until 80% to 90% confluence, and used at the third through fifth passages.

Pharmacological treatments and inhibitors

Endothelial cells were grown overnight in EBM-2 medium containing 1% albumax (LifeScience, Invitrogen). The next day, unless otherwise specified, cells were pre-incubated for 30 min with F₁-ATPase inhibitors (1 μ M IF1-H49K [18], or 125 nM oligomycin [27]), PI3K inhibitors (wortmannin, TGX-221 or AS-252424, 100 nM each) or a P2Y₁ receptor antagonist (1 μ M MRS2179), then treated for 15 min with apoA-I (100 μ g/mL), HDL (50 μ g/mL) or S1P (200 nM).

siRNA transfection

Endothelial cells were seeded at 150,000 cells/well in a 6-well plate in growth medium without antibiotics. They were then reverse transfected using lipofectamine® RNAimax (LifeScience, Invitrogen) in Optimem medium (Gibco) with 20 nM siRNA against PI3K β (CCCUUCGAUAAGAUAUUGAAUU (F) and UUCAUAUAUCUUAUCGAAGGGUU (R)), or P2Y₁ (AAGACAGGCUCCAGUUCUACUU (F) and GUAGAACUGGAAGCCUGUCUUUU (R)). 24 h later, a second transfection was performed and 4 h after that the medium was replaced with complete growth medium. After a further 24 h, cells were serum starved overnight before treatment. Silencing efficiency was determined by western blotting for PI3K β and by RT-qPCR for P2Y₁.

Western blot analysis

After treatment, cells were lysed in Laemmli buffer, boiled for 5 min at 100°C, then separated by SDS-PAGE and transferred onto PVDF membranes (Immobilon-P, Millipore). Immunodetection was achieved by incubating with the relevant primary antibodies in 5% BSA overnight at 4°C: anti-pAkt Ser⁴⁷³ (1:4000), anti-pAkt Thr³⁰⁸ (1:1000), anti-Akt (1:4000) or anti-PI3K β (1:1000). HRP-conjugated anti-rabbit (1:4000) secondary antibodies were incubated for 1 h at room temperature and immunoreactive proteins were detected with ECL reagents, according to the manufacturer's instructions. Membranes were analyzed using Chemidoc (Bio-Rad), and the densities of protein bands from western blot images were quantified using Image Lab software (Bio-Rad). Values of phosphorylated Akt were normalized against the amount of total Akt. Changes in phosphorylation levels were evaluated by comparing the differences between basal (untreated cells) and stimulated (treated cells) values and were expressed as fold increase over basal.

Reverse transcription-PCR (RT-PCR) and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted with the Trizol reagent (Invitrogen), then phase separation was performed with chloroform and RNA was precipitated with isopropanol. RNA concentrations were determined using a spectrophotometer. One microgram of total RNA was reverse-transcribed into first strand cDNA using hexamer. A minus-reverse transcriptase control (without the M-MLV reverse transcriptase enzyme) was performed and used as a negative control. For RT-qPCR, cDNA was then amplified by RT-qPCR with SYBR Green (Roche). The primers used to amplify RNA encoding PI3K β , P2Y₁ and the reference gene (ribosomal protein L27, Rpl27) were designed using the Primer Express software (Life Technologies SAS, Villebon sur Yvette, France) with the following sequences: Rpl27 (ATCGCCAAGAGATCAAAGATAA, TCTGAAGACATCCTTATTGACG), PI3K β (AGCTGGTTTGGATCTTCGGATG, CACAACITCAATGAGGCCAGAGC), and P2Y₁ (TCCACAAGGCATAGCCTCCAAG, AGCAAGAGTCTTCTTCACCATGAC). All real-time PCR reactions were performed on a LC480 (Roche), with the following thermal cycling parameters: annealing at 60°C for 40 s, amplification at 95°C for 10 s and dissociation at 95°C for 10 s. The PCR program was followed by a melting curve and values were normalized according to the amount of Rpl27. C_t values of Rpl27 were constant in all samples. Conventional PCR assays were carried out using the following primers: PI3K α (GTCAATCGGTGACTGTGTGG, GACGATCTCCAATTCCCAAA), PI3K β (TCAGCCTTCGCTCCTAATGT, TGCAAAGTCAGCAGGAAATG), PI3K γ (CAACGTTCCGGCAATAGTTT, CGACTGGTTTCTTCATGGT), P2Y₁ (CTGATACGTGGCATAAACC, GTTCAATTTGGCTCTGGCCG), P2Y₁₂ (TGGGAACAGGACCACTGAGA, GGGTGTAAAGGAATTCGGGCA), and P2Y₁₃ (CTCAGCAATGCCTTCTCTGGA, GGCCAGAAAGGTAGGCAAGT). PCR amplification was carried out on equal amounts of cDNA for 35 cycles for PI3K isoforms and 45 cycles for P2Y receptors, using the following parameters: initial denaturation step, 2 min at 95°C; amplification steps, 30 s at 58°C (PI3Ks) or 66°C (P2Ys) and 30 s at 72°C; and final elongation step, 30 min at 72°C. Amplification products were resolved on a 2% agarose gel and stained with ethidium bromide. Fragment sizes were determined by comparison with known DNA standards.

Proliferation assay

Cells were seeded at a density of 25,000 per 6-well plate and grown for 24 h in complete medium. Cells were then washed twice with PBS, incubated in control medium (EBM-2 + 2% FBS) and treated with or without apoA-I (100 μ g/mL), and with or without TGX-221 (100 nM) or MRS-2179 (1 μ M, twice a day). Cells were allowed to grow for a further 48 h, then were washed twice with PBS and harvested with trypsin to be counted with a Beckman Coulter Counter.

Statistical analysis

For the analysis of variance between groups of data, one-way ANOVA was performed followed by the Bonferroni test, using GraphPad Prism 5.0 (GraphPad Software). Results are expressed as means $\pm$ SEM, with differences considered significant at $p < 0.05$.

Results

ApoA-I induces Akt phosphorylation

To investigate whether the PI3K/Akt pathway is involved in signal transduction by apoA-I in endothelial cells (HUVECs), we analyzed the phosphorylation of Akt following

Fig. 1. ApoA-I and HDL induce Akt phosphorylation in HUVECs. (A, C) Kinetics experiments were performed on HUVECs following stimulation for 5 and 15 min with 100 μ g/ml apoA-I (A) or 50 μ g/ml HDL (C). (B, D) Dose response experiments were performed on HUVECs following stimulation for 15 min with apoA-I (B) or HDL (D) at 50 and 100 μ g/ml. (A, B, C, D) Cellular Akt phosphorylation was determined by western blot analysis using specific antibodies against phosphorylated Akt (pAkt Ser⁴⁷³ and pAkt Thr³⁰⁸). Equal loading was confirmed by reprobing membranes with anti-total Akt antibodies. Immunoblots shown are representative of 3 experiments.

Densities of protein bands from western blot images were quantified and pAkt Ser⁴⁷³ values were normalized against the amount of total Akt protein. Changes in phosphorylation were expressed as fold increase over basal values (untreated cells). Data are presented as means $\pm$ SEM (n = 3 per condition) and different subscripted letters indicate significant differences, tested by one-way ANOVA followed by the Bonferroni post-hoc test, with $p < 0.05$.

treatment with apoA-I and HDL. Kinetic and dose response experiments both revealed that apoA-I induced phosphorylation of Akt at its activation sites, threonine 308 (Thr308) and serine 473 (Ser473), within 5 min of stimulation (Fig. 1A), with a maximum phosphorylation observed at 100 μ g/ml (Fig. 1B). Similar effects were observed when cells were incubated with HDL (Fig. 1C, D). Our results show that, compared to unstimulated cells, the maximum increase in Akt Ser473 phosphorylation induced by apoA-I and HDL was 40 ± 2 and 46 ± 8 fold, respectively (Fig. 1B and 1D). These results indicate that apoA-I and HDL activate PI3K/Akt at similar levels.

Akt phosphorylation induced by apoA-I occurs through ecto-F₁-ATPase activation

We and others have found that ecto-F₁-ATPase is expressed at the cell surface of endothelial cells and acts as an apoA-I receptor [18–20, 28]. To investigate whether ecto-F₁-ATPase is involved in apoA-I-induced Akt phosphorylation, endothelial cells were treated with 1 μ M IF1-H49K, a selective protein inhibitor of F₁-ATPase activity, which has been previously shown to inhibit the effect of apoA-I on endothelial cell proliferation at this concentration [18]. As reported in Fig. 2A, cell treatment with IF1-H49K dramatically decreased (~93%) the phosphorylation of Akt induced by apoA-I (100 μ g/ml) whereas a low level of Akt phosphorylation persisted in HDL-stimulated cells treated by IF1-H49K. Interestingly, IF1-H49K did not affect S1P-induced phosphorylation of Akt (Fig. 2A). Since S1P activation involves S1P receptors [17], these results demonstrate that IF1-H49K specifically inhibits Akt phosphorylation induced by an apoA-I receptor. Similar results were also obtained with oligomycin (Fig. 2B), another inhibitor of F₁-ATPase activity [27, 29].

Fig. 2. Ecto- F_1 -ATPase is involved in apoA-I- and HDL-induced phosphorylation of Akt in HUVECs. (A) HUVECs were pre-treated or not with IF1-H49K (1 μ M) for 30 min then stimulated with apoA-I (100 μ g/ml), HDL (50 μ g/ml) or S1P (200 nM) for 15 min. (B) HUVECs were pre-treated or not with oligomycin (125 nM) for 30 min then stimulated with apoA-I (100 μ g/ml), HDL (50 μ g/ml) or S1P (200 nM) for 15 min. (A, B, C, D) The cellular phosphorylation of Akt was determined by western blot analysis using antibodies against phosphorylated Akt (pAkt Ser⁴⁷³). Equal loading was confirmed by reprobing membranes with anti-total Akt antibodies. Immunoblots shown are representative of 3 experiments. Phosphorylation levels of Ser⁴⁷³ were quantified and normalized against the amount of total Akt protein, then expressed as fold increase over untreated cells. Data are presented as means $\pm$ SEM ($n = 3$ per condition) and different subscripted letters indicate significant differences, tested by one-way ANOVA followed by the Bonferroni post-hoc test, with $p < 0.05$.

Altogether, these results indicate that Akt phosphorylation induced by apoA-I in endothelial cells is driven solely through ecto- F_1 -ATPase activity.

The P2Y₁ receptor is involved downstream of ecto- F_1 -ATPase activation by apoA-I

ApoA-I binding to ecto- F_1 -ATPase stimulates its ATPase activity and generates extracellular ADP [30]. We previously reported that ADP and its analogues (2MeSADP and ADP β S) but not its hydrolysis products (such as AMP and adenosine), promote endothelial cell survival in a similar manner to apoA-I [18], suggesting that extracellular ADP generated from the stimulation of ecto- F_1 -ATPase by apoA-I activates an ADP-sensitive P2Y receptor. We thus analyzed whether an ADP receptor of the P2Y family was involved in Akt phosphorylation downstream of F_1 -ATPase activation by apoA-I. P2Y receptors are G protein-coupled receptors (GPCR) and three of them are preferentially activated by ADP, namely P2Y₁, P2Y₁₂ and P2Y₁₃ [31, 32]. Due to the lack of specificity of all commercial antibodies tested against P2Y receptors, we analyzed the expression of P2Y₁, P2Y₁₂ and P2Y₁₃ receptors by assessing mRNA levels (Fig. 3A). As previously reported in HUVECs [33], we found that P2Y₁ mRNA was clearly detected, whereas P2Y₁₂ and P2Y₁₃ mRNA were barely detectable. As a control, and as previously reported [21], we observed that mRNA for P2Y₁ and P2Y₁₃, but not P2Y₁₂, were present in the hepatoma cell line HepG2 (Fig. 3A).

To assess whether the P2Y₁ receptor is involved in Akt phosphorylation downstream of ecto- F_1 -ATPase activation by apoA-I, HUVECs were pretreated with MRS2179, a selective P2Y₁ antagonist [33], before cell stimulation with apoA-I. Figure 3B shows that MRS2179 strongly decreased the phosphorylation of Akt induced by apoA-I. Furthermore, this effect was specific to apoA-I since Akt phosphorylation induced by S1P was unchanged following MRS2179 treatment (Fig. 3B). In order to ascertain the role of the P2Y₁ receptor in transducing apoA-I signaling, HUVECs were transfected with siRNA against P2Y₁ (Fig. 3C). The results show that P2Y₁ gene silencing (by up to 80%; Fig. 3B, inset), caused the complete inhibition of Akt phosphorylation induced by apoA-I (Fig. 3C). These data indicate that the P2Y₁ ADP

Fig. 3. The P2Y₁ receptor is involved in apoA-I-induced phosphorylation of Akt in HUVECs. (A) Total RNA was extracted from HUVECs and HepG2 cells to study the mRNA expression of the P2Y₁, P2Y₁₂ and P2Y₁₃ ADP receptors using classic RT-PCR. (B) HUVECs were pre-incubated for 30 min with 1 µM MRS2179 then stimulated with apoA-I (100 µg/ml) or S1P (200 nM) for 15 min. (C) HUVECs were transfected with siRNA control (si-control) or siRNA against P2Y₁ (si-P2Y₁), as described in the Materials and Methods section, and then stimulated with apoA-I (100 µg/ml) for 15 min. Inset: the efficiency of siRNA silencing in terms of gene expression levels. (B, C) Cellular Akt phosphorylation was determined by western blot analysis using antibodies against phosphorylated Akt (pAkt Ser⁴⁷³) and total Akt. Immunoblots shown are representative of 3 experiments. Phosphorylation levels of Ser⁴⁷³ were quantified, normalized against the amount of total Akt protein and then expressed as fold increase over untreated cells. Data are presented as means ± SEM (n = 3 per condition) and different subscripted letters indicate significant differences, tested by one-way ANOVA followed by the Bonferroni post-hoc test, with *p* < 0.05.

receptor is involved in Akt phosphorylation downstream of ecto-F₁-ATPase activation by apoA-I.

PI3K β mediates Akt phosphorylation induced by apoA-I

To further elucidate the signaling pathway mediating the effects of apoA-I activation in endothelial cells, we next sought to determine which isoform of PI3K is involved in apoA-I-induced Akt phosphorylation downstream of ecto-F₁-ATPase and P2Y₁ receptor activation. Endothelial cells have been shown to express the α , and γ isoforms of class I PI3Ks [34], and PCR analysis confirmed the presence of mRNA for these three isoforms in HUVECs (Fig. 4A). Quantitative real time PCR indicated that the α and β isoforms were highly expressed whereas the γ isoform was expressed to a lesser extent (not shown). We chose to specifically investigate the respective roles of the β and γ isoforms of PI3K in apoA-I-induced Akt phosphorylation since previous studies have shown that these are the main isoforms activated downstream of GPCR activation [35, 36]. Endothelial cells were treated with either wortmannin, a pan-PI3K inhibitor, or with selective PI3K β (TGX-221) or PI3K γ (AS-252424) inhibitors. We observed that the inhibition of PI3K β by TGX-221 abrogated the phosphorylation of Akt induced by apoA-I to the same extent as that of wortmannin, whereas the PI3K γ inhibitor did not affect Akt phosphorylation (Fig. 4B). Moreover, transfection of HUVECs with siRNA directed against PI3K p110 β (Fig. 4C) showed

Fig. 4. The β isoform of PI3K is involved in apoA-I-induced phosphorylation of Akt in HUVECs. (A) Total RNA was extracted from HUVECs to verify the expression of the α , β and γ isoforms of PI3K using classic RT-PCR. (B) HUVECs were pre-incubated for 30 min with TGX-221, wortmannin or AS-252424 at 100 nM, then stimulated with apoA-I (100 μ g/ml) for 15 min. (C) HUVECs were transfected with siRNA control (si-control) or siRNA against PI3K β (siPI3K β), as described in the Materials and Methods section, then stimulated with apoA-I (100 μ g/ml) for 15 min. The silencing efficiency of PI3K β is shown in the western blot. (B, C) Cellular Akt phosphorylation at Ser⁴⁷³ was determined by western blot analysis using antibodies against phosphorylated Akt (pAkt Ser⁴⁷³) and total Akt. Immunoblots shown are representative of 3 experiments. Phosphorylation levels of Ser⁴⁷³ were quantified, normalized against the amount of total Akt protein and then expressed as fold increase over untreated cells. Data are presented as means $\pm$ SEM ($n = 3$ per condition) and different subscripted letters indicate significant differences, tested by one-way ANOVA followed by the Bonferroni post-hoc test, with $p < 0.05$.

Fig. 5. The PI3K β isoform mediates endothelial cell proliferation induced by free apoA-I. HUVECs were grown for 24 h in complete medium, then incubated in EBM-2 medium + 2% FBS either alone (control) or with apoA-I (100 μ g/ml). Under each of these conditions, cells were also treated or not with the P2Y₁ antagonist MRS2179 (1 μ M, twice a day) or the PI3K β inhibitor TGX-221 (100 nM). Cells were grown for 48 h, washed twice with PBS, harvested with trypsin and then counted with a Beckman Coulter Counter. Values are expressed as the percentage of cells per well compared to the control condition. Data are presented as means $\pm$ SEM ($n = 6-9$ wells per group). Data with different subscripted letters indicate significant differences, tested by one-way ANOVA followed by the Bonferroni post-hoc test, with $p < 0.05$.

that silencing of the p110 β subunit of PI3K β (by up to 80%, assessed by western blotting, data not shown) completely inhibited apoA-I-induced Akt phosphorylation whereas a control siRNA had no effect. Altogether, these results demonstrate that PI3K β , and not PI3K γ , is involved in apoA-I-induced Akt phosphorylation in endothelial cells.

Pharmacological inhibition of the P2Y₁ receptor and PI3K β activity inhibits apoA-I-induced endothelial cell proliferation

Finally, we sought to establish the relevance of the P2Y₁/PI3K β axis downstream of apoA-I-induced ecto-F₁-ATPase activation by investigating its role in endothelial cell proliferation induced by apoA-I. HUVEC cells were pretreated with the P2Y₁ antagonist, MRS2179, the PI3K β inhibitor, TGX-221, or vehicle controls. The proliferation of cells grown in control medium alone (EBM-2 + 2% FBS, basal proliferation) was then compared with medium containing apoA-I (Fig. 5). As previously reported [18], we confirmed that adding apoA-I (100 μ g/mL) to control medium promoted endothelial cell proliferation by more than 20%. We then observed that MRS2179 and TGX-221 treatment prevented these positive effects of apoA-I on endothelial cell proliferation. Interestingly, TGX-221 inhibited cell proliferation (by 35%) independently of apoA-I stimulation, indicating that PI3K β activity contributed to the basal proliferation of endothelial cells. Conversely, endothelial cell proliferation mediated by P2Y₁ receptors was strictly dependent on apoA-I activation since MRS2179 had no effect on basal cell proliferation. Altogether, these results indicate that apoA-I activates the ecto-F₁-ATPase receptor to stimulate endothelial cell proliferation through activation of the P2Y₁ receptor and the PI3K β /Akt pathway.

Discussion

In this study, we have demonstrated that the PI3K/Akt signaling pathway is involved downstream of ecto-F₁-ATPase stimulation by apoA-I in HUVECs. ApoA-I binding to ecto-F₁-ATPase was previously shown to promote endothelial cell proliferation in an ADP-dependent manner [18], and we provide evidence here that signal transduction mediated by the apoA-I/ecto-F₁-ATPase axis depends specifically on the P2Y₁ ADP-receptor and involves the p110 β PI3K class I isoform (PI3K β).

Endothelial cells express several receptors that bind to HDL and their main apolipoprotein, apoA-I [7, 37]. Among these, ABCA1 and ecto-F₁-ATPase are known to bind to lipid-poor apoA-I with high affinity, whereas ABCG1 and SR-BI only interact with mature HDL particles [7, 18, 38]. Both ecto-F₁-ATPase and ABCA1 contribute towards HDL transcytosis [20, 39], an important process that depends on an intact endothelium and allows HDL to reach the subendothelial space of the arteries [7]. Besides their involvement in HDL transcytosis, ecto-F₁-ATPase and ABCA1 each have specific effects on endothelial cells. Notably, endothelial ABCA1 promotes cholesterol efflux to apoA-I and activates anti-inflammatory signaling pathways [7, 40, 41], (Vaisman et al. 2012) whereas ecto-F₁-ATPase activity promotes apoA-I-induced endothelial cell survival [18]. Furthermore, cholesterol efflux to HDL mediated by endothelial ABCG1 was shown to display non redundant functions than ABCA1 by promoting preservation of eNOS activity and suppression of endothelial inflammation *in vivo* [41]. Additionally, endothelial SR-BI signaling in response to HDL has been shown to involve the activation of Src and AMPK, which in turn activate the PI3K/Akt/MAPK pathways to facilitate secretion of vasodilating factors, including nitric oxide (NO) and prostacyclin (PGI₂), as well as endothelial cell proliferation and migration [16, 37]. The PI3K/Akt/MAPK signaling pathways are also activated in endothelial cells by sphingosine 1 phosphate (S1P), a bioactive phospholipid preferentially associated with apoM in HDL particles, which binds to endothelial S1P₁ and S1P₃ receptors. This has been shown to lead to eNOS activation and endothelial cell migration and survival [37, 42]. In the present work, we specifically targeted ecto-F₁-ATPase signaling by using lipid-free apoA-I, thereby excluding HDL-induced cell signaling through the SR-BI and S1P receptors. We show that lipid-free apoA-I activates a PI3K/Akt pathway that solely depends on the ecto-F₁-ATPase receptor, as evidenced by the complete inhibition of apoA-I-induced Akt phosphorylation by ecto-F₁-ATPase inhibitors. In addition to lipid-free apoA-I, ecto-F₁-ATPase binds to mature HDL particles [25, 30] and, accordingly, HDL-induced Akt phosphorylation was partially inhibited by ecto-F₁-ATPase inhibitors, with the remaining level of phosphorylated Akt likely

attributed to signaling downstream of SR-BI or S1P receptors. Thus, ecto-F₁-ATPase, SR-BI and S1P receptors all activate PI3K/Akt signaling pathways in response to HDL. This raises the question of whether these receptors cooperate or can compensate for each other in signal transduction induced by HDL. It has been suggested that the binding of HDL to SR-BI may provide a molecular platform for efficient receptor stimulation by HDL-associated S1P [37], but whether and how ecto-F₁-ATPase, SR-BI and S1P receptors interact in endothelial cells will need further investigation.

Previous studies have highlighted the close partnership between the stimulation of ecto-F₁-ATPase activity by apoA-I and P2Y ADP receptor signaling [32]. P2Y receptors are G protein-coupled receptors (GPCRs) that are activated by adenine and uridine nucleotides [43]. All of the three P2Y receptors that are preferentially activated by ADP (P2Y₁, P2Y₁₂ and P2Y₁₃) have been found to be coupled to ecto-F₁-ATPase activity and display different functions depending on the cell type [32]. For instance, in hepatocytes the apoA-I/ecto-F₁-ATPase/P2Y₁₃ axis activates an intracellular RhoA/ROCK1 signaling pathway that stimulates HDL uptake and appears to have atheroprotective properties by promoting reverse cholesterol transport [21–24, 30, 44, 45], and in adipocytes HDL-apoA-I, ecto-F₁-ATPase and P2Y signaling are all involved in lipid metabolism [46–50]. In endothelial cells, apoA-I/ecto-F₁-ATPase has been previously shown to stimulate HDL transcytosis *via* activation of P2Y₁₂ ADP receptors [20]. This study was performed on bovine aortic endothelial cells (BAECs) expressing both the P2Y₁ and P2Y₁₂ receptors and the authors used specific antagonists for each of these P2Y receptors to show that only P2Y₁₂ is involved in HDL transcytosis. In our study, we used HUVECs that express high levels of P2Y₁ but barely detectable levels of P2Y₁₂ and P2Y₁₃ (Fig. 3A and [33]), allowing us to specifically study P2Y₁ downstream of apoA-I/ecto-F₁-ATPase. Our data show that pharmacological inhibition and gene silencing of P2Y₁ fully inhibited apoA-I-induced Akt phosphorylation, indicating that the P2Y₁ receptor alone contributes to apoA-I/ecto-F₁-ATPase-induced PI3K/Akt signaling in this cell type. Moreover, apoA-I-induced cell proliferation was also completely blocked following pharmacological inhibition of the P2Y₁ receptor. It is thus likely that in endothelial cells ecto-F₁-ATPase is coupled to both the P2Y₁ and P2Y₁₂ receptors, the expression levels of which depend on the cell type, and that their activation promotes cell proliferation and HDL transcytosis respectively.

Some beneficial properties of endothelial P2Y₁ receptors in the vasculature have already been reported. For instance, using pharmacological approaches, different studies have evidenced that P2Y₁ contributes to ADP-induced vasodilatation of intact arteries through the release of dilating factors such as NO and PGI₂ [51, 52]. Furthermore, P2Y₁ receptors have been shown to promote ADP-mediated endothelial cell migration *in vitro* [33]. However, some other studies have suggested a role for endothelial P2Y₁ in adhesion molecule-dependent leucocyte recruitment in femoral arteries after the induction of acute inflammation by TNF α [53, 54]. Thus, P2Y₁ receptors may have opposite functions in vascular endothelial protection depending on the inflammatory status and stages of atherosclerotic development. In addition to these functions, we provide evidence for a role of endothelial P2Y₁ in cell proliferation downstream of ecto-F₁-ATPase activation by apoA-I. Our work has focused on identifying the intracellular signal transduction involved in this process and thus further investigation is required to determine whether activation of endothelial P2Y₁ by apoA-I/ecto-F₁-ATPase promotes other cellular responses, such as secretion of vasodilating factors and cell migration.

In terms of the cell signaling pathways downstream of apoA-I-induced ecto-F₁-ATPase/P2Y₁ activation, we found here that it specifically involves the class I PI3K (PI3K) β isoform. This result is supported by a recent study demonstrating that endothelial PI3K β promotes endothelial repair *in vivo* [55]. PI3K β is composed of a p110 catalytic and a p85 regulatory subunit, the latter being activated by tyrosine kinase receptors [56], however this kinase seems to be preferentially activated downstream of GPCR activation [36]. Accordingly, our study demonstrated that the PI3K β isoform - and not the γ isoform - is responsible for Akt phosphorylation and cell proliferation in apoA-I-stimulated endothelial cells. It has been

proposed that activation of PI3K β requires the synergistic activation of both the β and γ G protein subunits as well as a phosphotyrosyl peptide [57, 58]. We cannot exclude the possibility that, in endothelial cells, apoA-I stimulation induces tyrosine kinase activation through ecto-F₁-ATPase to recruit PI3K β downstream of P2Y₁ receptor activation, but the nature of this tyrosine kinase remains to be identified. PI3K β has also been reported to be essential in S1P-stimulated endothelial cells [59, 60], suggesting that this kinase is a central protein in HDL-induced endothelial cell proliferation through its role downstream of different receptors, such as S1P receptors or ecto-F₁-ATPase. Moreover, PI3K β has been reported to mediate NO production in endothelial cells following the activation of different receptors [59, 61]. As mentioned above, both HDL and P2Y₁ can also contribute to NO secretion in endothelial cells and it could thus be of great interest to further investigate the possible involvement of the HDL/P2Y₁/PI3K β axis in eNOS activation and the respective contributions of these different HDL receptors in this process.

Recently, class II PI3Ks have also been demonstrated to be important for HDL signaling. These PI3Ks have been investigated to a much lesser extent than the class I PI3Ks and their roles are not well understood [62], however it seems that class II PI3Ks play an important function in HDL signaling downstream of S1P receptor activation. Although the molecular mechanisms leading to the activation of class II PI3Ks are not clearly understood, it was reported that GPCRs can activate this class of PI3K [62]. Therefore, we cannot exclude the possibility that, through activation of a P2Y receptor, apoA-I could also activate a class II PI3K that regulates other biological processes such as endothelial cell migration and survival.

The number of different PI3K isoforms led us to question whether each isoform has a different role within endothelial cells. This question is particularly relevant since pharmaceutical industries have started to develop isoform-specific PI3K inhibitors. Our results clearly identify that PI3K γ is not involved downstream of apoA-I-induced cell signaling mediated through endothelial ecto-F₁-ATPase. PI3K γ is a well-known mediator of inflammation in arterial walls through its roles in both immune cell signaling [63, 64] and in endothelial cells where it can modulate the E-selectin pathway necessary for leucocyte recruitment [65]. The identification of a beneficial role of PI3K β in endothelial cells confirms the specific role of these different PI3K isoforms in vascular diseases [34] and demonstrates that these two isoforms of PI3K may play opposite roles in a physiopathological context and especially in vascular diseases. PI3K is known to have an important role in platelet aggregation, and specific PI3K β inhibitors prevent the formation of stable $\alpha_{IIb}\beta_3$ integrin adhesion contacts, leading to defective platelet thrombus formation. *In vivo*, the use of these inhibitors eliminates occlusive thrombus formation but does not prolong bleeding time [66–68], identifying the PI3K β isoform as a useful target in the development of antithrombotic therapies. Since our study suggests that PI3K β is also important for endothelial protection induced by HDL-apoA-I, it seems necessary to now evaluate the *in vivo* effects of PI3K β inhibition on re-endothelialization before proposing this kinase as a valid pharmacological target for antithrombotic therapy in patients.

To conclude, the present study demonstrates that signal transduction downstream of endothelial ecto-F₁-ATPase activation by HDL-apoA-I occurs through the specific activation of the P2Y₁ ADP receptor and involves a PI3K β /Akt signaling pathway. Moreover, we have identified that this signaling module is responsible for HDL-apoA-I-induced endothelial cell proliferation. Thus, this work has identified a novel signaling pathway which may contribute to the atheroprotective functions of HDL-apoA-I. Future *in vivo* research must now evaluate whether this pathway contributes to the repair, or re-endothelialization, of an endothelial lesion.

Acknowledgements

This work was supported by grants from the French National Research Agency (ANR, #ANR-16-CE18-0014-01, HDL-NEXT-THERAPEUTICS) and the "Région Midi-Pyrénées -

Occitanie” (CLE N° 14054132, RBIO N° 14054337). ACB was a recipient of a thesis fellowship from the “Région Midi-Pyrénées”.

Disclosure Statement

The authors declare no conflict of interest.

References

- 1 Forte A, Rinaldi B, Berrino L, Rossi F, Galderisi U, Cipollaro M: Novel potential targets for prevention of arterial restenosis: insights from the pre-clinical research. *Clin Sci (Lond)* 2014;127:615–634.
- 2 Zhu T, Yao Q, Hu X, Chen C, Yao H, Chao J: The Role of MCP1 in Ischemia/Reperfusion Injury-Induced HUVEC Migration and Apoptosis. *Cell Physiol Biochem* 2015;37:577–591.
- 3 Chapman MJ, Ginsberg HN, Amarencu P, Andreotti F, Borén J, Catapano AL, Descamps OS, Fisher E, Kovanen PT, Kuivenhoven JA, Lesnik P, Masana L, Nordestgaard BG, Ray KK, Reiner Z, Taskinen M-R, Tokgözoğlu L, Tybjaerg-Hansen A, Watts GF: Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. *Eur Heart J* 2011;32:1345–1361.
- 4 Rye K-A, Barter PJ: Cardioprotective functions of HDLs. *J Lipid Res* 2014;55:168–79.
- 5 Kratzer A, Giral H, Landmesser U: High-density lipoproteins as modulators of endothelial cell functions: alterations in patients with coronary artery disease. *Cardiovasc Res* 2014;103:350–361.
- 6 Riwayto M, Landmesser U: High density lipoproteins and endothelial functions: mechanistic insights and alterations in cardiovascular disease. *J Lipid Res* 2013;54:3227–3243.
- 7 Tran-Dinh A, Diallo D, Delbosc S, Varela-Perez LM, Dang QB, Lapergue B, Burillo E, Michel JB, Levoe A, Martin-Ventura JL, Meilhac O: HDL and endothelial protection. *Br J Pharmacol* 2013;169:493–511.
- 8 von Eckardstein A, Rohrer L: HDLs in crises. *Curr Opin Lipidol* 2016;27:264–273.
- 9 Strong A, Rader DJ: Clinical Implications of Lipid Genetics for Cardiovascular Disease. *Curr Cardiovasc Risk Rep* 2010;4:461–468.
- 10 Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, Hindy G, Hólm H, Ding EL, Johnson T, Schunkert H, Samani NJ, Clarke R, Hopewell JC, Thompson JE, Li M, Thorleifsson G, Newton-Cheh C, Musunuru K, Pirruccello JP, Saleheen D, Chen L, Stewart AFR, Schillert A, Thorsteinsdottir U, Thorgeirsson G, Anand S, Engert JC, Morgan T, Spertus J, et al.: Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet* 2012;380:572–580.
- 11 Verdier C, Martinez LO, Ferrières J, Elbaz M, Genoux A, Perret B: Targeting high-density lipoproteins: update on a promising therapy. *Arch Cardiovasc Dis* 2013;106:601–611.
- 12 Riwayto M, Rohrer L, von Eckardstein A, Landmesser U: Dysfunctional HDL: from structure-function-relationships to biomarkers. *Handb Exp Pharmacol* 2015;224:337–366.
- 13 Tauber JP, Cheng J, Gospodarowicz D: Effect of high and low density lipoproteins on proliferation of cultured bovine vascular endothelial cells. *J Clin Invest* 1980;66:696–708.
- 14 Tauber JP, Cheng J, Massaglia S, Gospodarowicz D: High density lipoproteins and the growth of vascular endothelial cells in serum-free medium. *In Vitro* 1981;17:519–530.
- 15 Suc I, Escargueil-Blanc I, Trolly M, Salvayre R, Negre-Salvayre A: HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL. *Arter Thromb Vasc Biol* 1997;17:2158–2166.
- 16 Seetharam D, Mineo C, Gormley AK, Gibson LL, Vongpatanasin W, Chambliss KL, Hahner LD, Cummings ML, Kitchens RL, Marcel YL, Rader DJ, Shaul PW: High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circ Res* 2006;98:63–72.
- 17 Kimura T, Sato K, Malchinkhuu E, Tomura H, Tamama K, Kuwabara A, Murakami M, Okajima F: High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors. *Arter Thromb Vasc Biol* 2003;23:1283–1288.
- 18 Radojkovic C, Genoux A, Pons V, Combes G, de Jonge H, Champagne E, Rolland C, Perret B, Collet X, Terce F, Martinez LO: Stimulation of Cell Surface F1-ATPase Activity by Apolipoprotein A-I Inhibits Endothelial Cell Apoptosis and Promotes Proliferation. *Arter Thromb Vasc Biol* 2009;29:1125–1130.

- 19 González-Pecchi V, Valdés S, Pons V, Honorato P, Martinez LO, Lamperti L, Aguayo C, Radojkovic C: Apolipoprotein A-I enhances proliferation of human endothelial progenitor cells and promotes angiogenesis through the cell surface ATP synthase. *Microvasc Res* 2015;98:9–15.
- 20 Cavalier C, Ohnsorg PM, Rohrer L, von Eckardstein A: The beta-Chain of Cell Surface F0F1 ATPase Modulates ApoA-I and HDL Transcytosis Through Aortic Endothelial Cells. *Arter Thromb Vasc Biol* 2012;32:131–139.
- 21 Jacquet S, Malaval C, Martinez LO, Sak K, Rolland C, Perez C, Nauze M, Champagne E, Terce F, Gachet C, Perret B, Collet X, Boeynaems JM, Barbaras R: The nucleotide receptor P2Y13 is a key regulator of hepatic high-density lipoprotein (HDL) endocytosis. *Cell Mol Life Sci* 2005;62:2508–2515.
- 22 Fabre ACC, Malaval C, Ben Addi A, Verdier C, Pons V, Serhan N, Lichtenstein L, Combes G, Huby T, Briand F, Collet X, Nijstad N, Tietge UJF, Robaye B, Perret B, Boeynaems J-MM, Martinez LO: P2Y13 receptor is critical for reverse cholesterol transport. *Hepatology* 2010;52:1477–1483.
- 23 Lichtenstein L, Serhan N, Espinosa-Delgado S, Fabre A, Annema W, Tietge UJF, Robaye B, Boyenaems J-M, Laffargue M, Perret B, Martinez LO: Increased Atherosclerosis in P2Y13 / Apolipoprotein E Double-Knockout Mice: Contribution of P2Y13 to Reverse Cholesterol Transport. *Cardiovasc Res* 2015;106:315–323.
- 24 Goffinet M, Tardy C, Boubekeur N, Cholez G, Bluteau GA, Oniciu DC, Lalwani ND, Dasseux J-LH, Barbaras R, Baron R: P2Y13 Receptor Regulates HDL Metabolism and Atherosclerosis In Vivo. *PLoS One* 2014;9:e95807.
- 25 Martinez LO, Georgeaud V, Rolland C, Collet X, Tercé F, Perret B, Barbaras R: Characterization of Two High-Density Lipoprotein Binding Sites on Porcine Hepatocyte Plasma Membranes: Contribution of Scavenger Receptor Class B Type I (SR-BI) to the Low-Affinity Component. *Biochemistry* 2000;39:1076–1082.
- 26 Cabezon E, Butler PJ, Runswick MJ, Walker JE: Modulation of the oligomerization state of the bovine F1-ATPase inhibitor protein, IF1, by pH. *J Biol Chem* 2000;275:25460–25464.
- 27 Fabre AC, Vantourout P, Champagne E, Terce F, Rolland C, Perret B, Collet X, Barbaras R, Martinez LO: Cell surface adenylate kinase activity regulates the F1-ATPase/P2Y13-mediated HDL endocytosis pathway on human hepatocytes. *Cell Mol Life Sci* 2006;63:2829–2837.
- 28 Moser TL, Stack MS, Asplin I, Enghild JJ, Hojrup P, Everitt L, Hubchak S, Schnaper HW, Pizzo S V: Angiostatin binds ATP synthase on the surface of human endothelial cells. *Proc Natl Acad Sci U S A* 1999;96:2811–2816.
- 29 Linnett PE, Beechey RB: Inhibitors of the ATP synthetase system. *Methods Enzym* 1979;55:472–518.
- 30 Martinez LO, Jacquet S, Esteve JP, Rolland C, Cabezon E, Champagne E, Pineau T, Georgeaud V, Walker JE, Terce F, Collet X, Perret B, Barbaras R: Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. *Nature* 2003;421:75–79.
- 31 Martinez LO, Cabou C, Pons V, Malaval C: P2Y receptors in atherosclerosis: from lipid metabolism to vascular functions. *WIREs Membr Transp Signal* 2012;1:743–754.
- 32 Martinez LO, Najib S, Perret B, Cabou C, Lichtenstein L: Ecto-F1-ATPase/P2Y pathways in metabolic and vascular functions of high density lipoproteins. *Atherosclerosis* 2015;238:89–100.
- 33 Shen J, DiCorleto PE: ADP stimulates human endothelial cell migration via P2Y1 nucleotide receptor-mediated mitogen-activated protein kinase pathways. *Circ Res* 2008;102:448–456.
- 34 Lupieri A, Smirnova N, Malet N, Gayral S, Laffargue M: PI3K signaling in arterial diseases: Non redundant functions of the PI3K isoforms. *Adv Biol Regul* 2015;59:4–18.
- 35 Wymann MP, Zvelebil M, Laffargue M: Phosphoinositide 3-kinase signalling--which way to target? *Trends Pharmacol Sci* 2003;24:366–376.
- 36 Guillermet-Guibert J, Bjorklof K, Salpekar A, Gonella C, Ramadani F, Bilancio A, Meek S, Smith AJH, Okkenhaug K, Vanhaesebroeck B: The p110beta isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110gamma. *Proc Natl Acad Sci U S A* 2008;105:8292–8297.
- 37 Nofer J-R: Signal transduction by HDL: agonists, receptors, and signaling cascades. *Handb Exp Pharmacol* 2015;224:229–256.
- 38 Martinez LO, Jacquet S, Terce F, Collet X, Perret B, Barbaras R: New insight on the molecular mechanisms of high-density lipoprotein cellular interactions. *Cell Mol Life Sci* 2004;61:2343–2360.
- 39 Cavalier C, Rohrer L, von Eckardstein A: ATP-Binding cassette transporter A1 modulates apolipoprotein A-I transcytosis through aortic endothelial cells. *Circ Res* 2006;99:1060–1066.

- 40 Vaisman BL, Demosky SJ, Stonik JA, Ghias M, Knapper CL, Sampson ML, Dai C, Levine SJ, Remaley AT: Endothelial expression of human ABCA1 in mice increases plasma HDL cholesterol and reduces diet-induced atherosclerosis. *J Lipid Res* 2012;53:158–167.
- 41 Westerterp M, Tsuchiya K, Tattersall IW, Fotakis P, Bochem AE, Molusky MM, Ntonga V, Abramowicz S, Parks JS, Welch CL, Kitajewski J, Accili D, Tall AR: Deficiency of ATP-Binding Cassette Transporters A1 and G1 in Endothelial Cells Accelerates Atherosclerosis in Mice. *Arterioscler Thromb Vasc Biol* 2016;36:1328–1337.
- 42 Argraves KM, Argraves WS: HDL serves as a S1P signaling platform mediating a multitude of cardiovascular effects. *J Lipid Res* 2007;48:2325–2333.
- 43 Boeynaems J-M, Communi D, Robaye B: Overview of the pharmacology and physiological roles of P2Y receptors. *WIREs Membr Transp Signal* 2012;1:581–588.
- 44 Malaval C, Laffargue M, Barbaras R, Rolland C, Peres C, Champagne E, Perret B, Tercé F, Collet X, Martinez LO: RhoA/ROCK I signalling downstream of the P2Y₁₃ ADP-receptor controls HDL endocytosis in human hepatocytes. *Cell Signal* 2009;21:120–127.
- 45 Blom D, Yamin T-T, Champy M-F, Selloum M, Bedu E, Carballo-Jane E, Gerckens L, Luell S, Meurer R, Chin J, Mudgett J, Puig O: Altered lipoprotein metabolism in P2Y₁₃ knockout mice. *Biochim Biophys Acta* 2010;1801:1349–1360.
- 46 Laplante MA, Monassier L, Freund M, Bousquet P, Gachet C: The purinergic P2Y₁ receptor supports leptin secretion in adipose tissue. *Endocrinology* 2010;151:2060–2070.
- 47 Lee H, Jun DJ, Suh BC, Choi BH, Lee JH, Do MS, Suh BS, Ha H, Kim KT: Dual roles of P2 purinergic receptors in insulin-stimulated leptin production and lipolysis in differentiated rat white adipocytes. *J Biol Chem* 2005;280:28556–28563.
- 48 Kim BW, Choo HJ, Lee JW, Kim JH, Ko YG: Extracellular ATP is generated by ATP synthase complex in adipocyte lipid rafts. *Exp Mol Med* 2004;36:476–485.
- 49 Arakaki N, Kita T, Shibata H, Higuti T: Cell-surface H⁺-ATP synthase as a potential molecular target for anti-obesity drugs. *FEBS Lett* 2007;581:3405–3409.
- 50 Howard AD, Verghese PB, Arrese EL, Soulages JL: The beta-subunit of ATP synthase is involved in cellular uptake and resecretion of apoA-I but does not control apoA-I-induced lipid efflux in adipocytes. *Mol Cell Biochem* 2011;348:155–164.
- 51 Guns PJ, Korda A, Crauwels HM, Assche T Van, Robaye B, Boeynaems JM, Bult H: Pharmacological characterization of nucleotide P2Y receptors on endothelial cells of the mouse aorta. *Br J Pharmacol* 2005;146:288–295.
- 52 Wihlborg A-K, Malmjö M, Eyjolfsson A, Gustafsson R, Jacobson K, Erlinge D: Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor. *Br J Pharmacol* 2003;138:1451–1458.
- 53 Hechler B, Freund M, Ravanat C, Magnenat S, Cazenave JP, Gachet C: Reduced atherosclerotic lesions in P2Y₁/apolipoprotein E double-knockout mice: the contribution of non-hematopoietic-derived P2Y₁ receptors. *Circulation* 2008;118:754–763.
- 54 Zerr M, Hechler B, Freund M, Magnenat S, Lanois I, Cazenave JP, Leon C, Gachet C: Major contribution of the P2Y₁ receptor in purinergic regulation of TNF α -induced vascular inflammation. *Circulation* 2011;123:2404–2413.
- 55 Haddad G, Zhabyeyev P, Farhan M, Zhu LF, Kassiri Z, Rayner DC, Vanhaesebroeck B, Oudit GY, Murray AG: Phosphoinositide 3-kinase β mediates microvascular endothelial repair of thrombotic microangiopathy. *Blood* 2014;124:2142–2149.
- 56 Logan SK, Falasca M, Hu P, Schlessinger J: Phosphatidylinositol 3-kinase mediates epidermal growth factor-induced activation of the c-Jun N-terminal kinase signaling pathway. *Mol Cell Biol* 1997;17:5784–5790.
- 57 Murga C, Fukuhara S, Gutkind JS: A novel role for phosphatidylinositol 3-kinase beta in signaling from G protein-coupled receptors to Akt. *J Biol Chem* 2000;275:12069–12073.
- 58 Kurosu H, Maehama T, Okada T, Yamamoto T, Hoshino S, Fukui Y, Ui M, Hazeki O, Katada T: Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110 β is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. *J Biol Chem* 1997;272:24252–24256.
- 59 Igarashi J, Michel T: Sphingosine 1-phosphate and isoform-specific activation of phosphoinositide 3-kinase beta. Evidence for divergence and convergence of receptor-regulated endothelial nitric-oxide synthase signaling pathways. *J Biol Chem* 2001;276:36281–36288.

- 60 Heller R, Chang Q, Ehrlich G, Hsieh SN, Schoenwaelder SM, Kuhlencordt PJ, Preissner KT, Hirsch E, Wetzker R: Overlapping and distinct roles for PI3K β and gamma isoforms in S1P-induced migration of human and mouse endothelial cells. *Cardiovasc Res* 2008;80:96–105.
- 61 Uruno A, Sugawara A, Kanatsuka H, Kagechika H, Saito A, Sato K, Kudo M, Takeuchi K, Ito S.: Upregulation of nitric oxide production in vascular endothelial cells by all-trans retinoic acid through the phosphoinositide 3-kinase/Akt pathway. *Circulation* 2005;112:727–736.
- 62 Tibolla G, Piñeiro R, Chiozzotto D, Mavrommati I, Wheeler AP, Norata GD, Catapano AL, Maffucci T, Falasca M: Class II phosphoinositide 3-kinases contribute to endothelial cells morphogenesis. *PLoS One* 2013;8:e53808.
- 63 Fougerat A, Gayral S, Gourdy P, Schambourg A, Ruckle T, Schwarz MK, Rommel C, Hirsch E, Arnal JF, Salles JP, Perret B, Breton-Douillon M, Wymann MP, Laffargue M: Genetic and pharmacological targeting of phosphoinositide 3-kinase-gamma reduces atherosclerosis and favors plaque stability by modulating inflammatory processes. *Circulation* 2008;117:1310–1317.
- 64 Smirnova NF, Gayral S, Pedros C, Loirand G, Vaillant N, Malet N, Kassem S, Calise D, Goudounèche D, Wymann MP, Hirsch E, Gadeau A-P, Martinez LO, Saoudi A, Laffargue M: Targeting PI3K γ activity decreases vascular trauma-induced intimal hyperplasia through modulation of the Th1 response. *J Exp Med* 2014;211:1779–1792.
- 65 Puri KD, Doggett TA, Huang C-Y, Douangpanya J, Hayflick JS, Turner M, Penninger J, Diacovo TG: The role of endothelial PI3K γ activity in neutrophil trafficking. *Blood* 2005;106:150–157.
- 66 Jackson SP, Schoenwaelder SM, Goncalves I, Nesbitt WS, Yap CL, Wright CE, Kenche V, Anderson KE, Dopheide SM, Yuan Y, Sturgeon SA, Prabakaran H, Thompson PE, Smith GD, Shepherd PR, Daniele N, Kulkarni S, Abbott B, Saylik D, Jones C, Lu L, Giuliano S, Hughan SC, Angus JA, Robertson AD, Salem HH: PI 3-kinase p110 β : a new target for antithrombotic therapy. *Nat Med* 2005;11:507–514.
- 67 Canobbio I, Stefanini L, Cipolla L, Ciraolo E, Gruppi C, Balduini C, Hirsch E, Torti M: Genetic evidence for a predominant role of PI3K β catalytic activity in ITAM- and integrin-mediated signaling in platelets. *Blood* 2009;114:2193–2196.
- 68 Martin V, Guillermet-Guibert J, Chicanne G, Cabou C, Jandrot-Perrus M, Plantavid M, Vanhaesebroeck B, Payrastre B, Gratacap M-P: Deletion of the p110 β isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo. *Blood* 2010;115:2008–2013.