sPLA2-V INHIBITS EPCR ANTICOAGULANT AND ANTIAPOPTOTIC PROPERTIES BY ACCOMMODATING LYSOPHOSPHATIDYLCHOLINE OR PAF IN THE HYDROPHOBIC GROOVE

Jacinto López-Sagaseta[1,#], Cristina Puy[1,#], Mikel Allende[1], Jorge Cerveró[1], Susana E. Velasco[1], Charles T. Esmon[2], Ramón Montes[1], José Hermida[1*]

[1]Division of Cardiovascular Sciences, Laboratory of Thrombosis and Haemostasis, Centre for Applied Medical Research, University of Navarra, Pamplona, Spain.

[2]The Howard Hughes Medical Institute, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA

[#]These authors share first authorship.

Present address for J.L-S.: Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL.

[*]**Corresponding author:** José Hermida,Laboratory of Thrombosis and Haemostasis, Division of Cardiovascular Sciences, Centre for Applied Medical Research (CIMA), University of Navarra.

Avenida Pío XII 55, 31008 Pamplona, Spain. e-mail: jhermida@unav.es

Phone: +34948194700; Fax: +34948194716;

Short title: EPCR inhibition by sPLA2-V-produced lipids.

Scientific Heading: Thrombosis and Hemostasis

Keywords: Endothelial cell protein C receptor; secretory group V phospholipase A2; phosphatidylcholine; lysophosphatidylcholine; platelet activating factor

Word count: 3,821

ABSTRACT

The endothelial protein C receptor (EPCR) plays an important role in cardiovascular disease by binding protein C/activated protein C (APC). EPCR structure contains a hydrophobic groove filled with an unknown phospholipid needed to perform its function. It is unknown whether lipid exchange takes place in EPCR as a regulatory mechanism of its activity. Our objective was to identify this phospholipid and to explore the possibility of a lipid exchange as a regulatory mechanism of EPCR activity driven by the endothelially expressed secretory group V phospholipase A2 (sPLA2-V). We identified phosphatidylcholine (PCh) as the major phospholipid bound to human soluble EPCR (sEPCR). PCh in EPCR could be exchanged by lysophosphatidylcholine (lysoPCh) and platelet activating factor (PAF). Remarkably, lysoPCh and PAF impaired the protein C binding ability of sEPCR. Inhibition of sPLA2-V, responsible for lysoPCh and PAF generation, improved APC binding to endothelial cells. Accordingly, EPCR-dependent protein C activation and APC antiapoptotic effect were significantly enhanced. Endothelial cell supplementation with sPLA2-V resulted in the opposite effects. In summary, EPCR accommodates PCh within its hydrophobic groove. LysoPCh and PAF impairing EPCR binding properties. sPLA2-V, as a source of these lipids, emerges as a potential therapeutic target for patients with inflammatory and thrombotic diseases.

INTRODUCTION

The endothelial cell protein C/activated protein C receptor (EPCR) is a transmembrane glycoprotein that is abundantly expressed on the surface of the endothelium of the large vessels.[1] EPCR binds protein C, activated protein C (APC), and factor VII/VIIa (FVII/VIIa) with high affinity.[2,3] Upon EPCR binding on the endothelial surface, protein C activation by the thrombin-thrombomodulin complex is notably increased and factor Xa-dependent FVII activation is reduced.[4,5] EPCR is a cornerstone molecule in the protein C system which plays an important role in cardiovascular pathologies such as thrombosis, coronary artery disease and stroke,[6-8] and is involved in mechanisms of vascular biology such as inflammation and endothelial barrier protection related to sepsis, lung function and metastasis.[9,10]

Previous structural studies showed that EPCR folds into two alpha chains which, together with a β-sheet platform, conform a hydrophobic groove. This is occupied by a phospholipid which has not yet been identified but which is necessary to preserve the ligand binding properties of EPCR.[11] One of the aims of the present work was to identify the exact nature of this phospholipid. Since EPCR shares significant homology with CD1d and MHC-class I like molecules which behave as lipid antigen-presenting molecules,[12] we speculated that the EPCR hydrophobic pocket could be filled with more than one phospholipid. We provide evidence that fully active EPCR accommodates phosphatidylcholine (PCh) within its hydrophobic groove and that, interestingly, secretory group V phospholipase A2 (sPLA2-V) generates bioactive lipids i.e. lysophosphatidylcholine (lysoPCh) and platelet activating factor (PAF) in the vicinity of EPCR which, by displacing phosphatidylcholine from the hydrophobic groove, notably impair the ability of EPCR to interact with protein C and FVII.

MATERIALS AND METHODS

An expanded version is available in the Supplemental Methods section.

Recombinant proteins expression and purification

Human and murine yeast-derived sEPCR as well as human mammalian cell-derived sEPCR were expressed and purified as described.[13-15]

Lipid extraction from sEPCR variants and subsequent lipid identification

The lipid fraction of sEPCR was extracted for subsequent analysis by thin layer chromatography (TLC) and mass spectrometry analysis.

Lipid extraction for subsequent analysis of sEPCR function

n-octyl-β-d-Glucopyranoside was used to extract the lipid from the yeast-derived sEPCR. The delipidated sEPCR was subsequently used for surface plasmon resonance (SPR) and fluorescence spectroscopy analyses as explained below.

Assessment of the interaction between lipids and sEPCR by fluorescence spectroscopy analysis

The binding of PCh, lysoPCh, PAF and S1P to human delipidated sEPCR was studied by measuring the changes in the intrinsic fluorescence of delipidated sEPCR upon incubation with increasing amounts of the lipids. The data were used to calculate the dissociation constant (K_D) for each lipid.

Assessment of the interaction between lysoPCh or PAF and sEPCR by mass spectrometry

Each lipid was incubated with sEPCR at 10-fold excess. Once the unbound lipid was removed by gel exclusion chromatography, the lipid fraction of the eluted protein was extracted and analyzed by mass spectrometry.

Biomolecular interaction analyses by SPR

All interaction experiments were performed by SPR technology using a BIAcore X Biosensor (GE Healthcare) basically as previously described.[3]

Flow cytometry experiments

All the flow cytometry experiments were performed in a FACScalibur (BD Biosciences). The binding of APC to endothelial cells was performed as described.[3] The expression of EPCR, thrombomodulin and sPLA2-V on the surface of cells was assessed using anti-human EPCR RCR-2, anti-human thrombomodulin and anti-human sPLA2-V monoclonal antibodies (mAbs) respectively. Apoptosis of staurosporine-stimulated endothelial cells was assessed by incubating them with fluorescently-labeled annexin V and actinomicine D.

Activation of protein C on the endothelial surface

The effect of manoalide and $sPLA_2$-V on APC generation by thrombin on the surface of HAEC or EA.hy926 was studied as previously described.[13]

Immunohistochemical assessment of sPLA2-V expression in tissues

Sections from carotid arteries from mice subjected to a laser injury-induced thrombosis[16] and from human left atrial appendages were analyzed for the presence of sPLA2-V using an anti-sPLA2-V Ab followed by the anti-rabbit Dako Envision+ System-HRP, and for the presence of fibrin deposits using Masson's Trichrome staining.

RESULTS

PCh is the phospholipid located in the hydrophobic pocket of EPCR

The lipid fraction extracted from yeast-expressed human sEPCR migrated in the TLC plate as a single spot exactly as PCh standard did. The same result was obtained using human sEPCR produced in mammalian cells, thus suggesting that the lipid naturally occupying the pocket of human sEPCR is PCh (Figure 1A). The experiment performed with murine sEPCR revealed a major spot corresponding to PCh and a minor one which migrates like PE.

To further analyze the nature of the EPCR lipid content, the sEPCR organic extract was subjected to mass spectrometry analysis which showed a peak at m/z 786, whose fragmentation yielded a pattern of peaks that are characteristic of the PCh polar group (Figure 1B). Commercially available PCh [molecular weight (MW) = 786 Da] rendered the same results, as did the lipid extracted from murine sEPCR. Finally, two peaks corresponding to two PCh molecules of different MW (786 and 758 Da) were obtained testing human sEPCR produced in mammalian cells, and both of them exhibited the characteristic peak pattern of the PCh polar group fragmentation. Collectively, these analyses demonstrate that the main lipid molecule bound to human EPCR is PCh.

PCh is essential for EPCR to interact with its ligands

We used SPR technology to analyze whether PCh was necessary to preserve the ability of sEPCR to interact with APC. The affinity of EPCR for APC was around 10-times lower when PCh was removed from sEPCR (K_D = 64 ± 8 and 616 ± 156 nM with and without PCh respectively, n = 3) (Figure 2A, 2B), a result that is coherent with previous findings.[11] The ability of APC to bind to delipidated sEPCR was fully recovered after incubation with PCh (Figure 2C). Of note, the K_D was 62 ± 27 nM (n = 3), very similar to the obtained with non-

manipulated sEPCR. Delipidated sEPCR also lost the ability to bind to FVIIa (K_D = 83 ± 16 and 625 ± 147 nM with and without PCh respectively).

LysoPCh and PAF but not S1P can locate in the hydrophobic pocket of EPCR

Since lysoPCh is derived from PCh and preserves the structure of the latter except that it lacks one of the fatty acid groups (Supplemental Figure 1), we speculated that EPCR could accommodate it. For this reason we used fluorescence spectroscopy to monitor whether the intrinsic fluorescence of delipidated sEPCR changed upon incubation with lysoPCh. As can be seen, the three lysoPCh forms tested did modify the intrinsic fluorescence in a dose-dependent manner (Figure 3A). As a reference, changes in the intrinsic fluorescence of delipidated sEPCR were also monitored upon PCh (MW = 760 Da) addition. Interestingly, delipidated sEPCR interacted more strongly with all lysoPCh forms than with PCh as shown by the K_D obtained for each condition: 7.5 ± 8.5, 3.6 ± 1.0, 1.1 ± 0.2 and 108.4 ± 54.5 μM for lysoPCh 16:0, 18:0, 18:1 and PCh respectively. Bearing in mind the similarity of PAF and to a lesser extent S1P to PCh and LysoPCh (Supplemental Figure 1), we wondered if EPCR could accommodate either of these molecules. S1P did not modify the intrinsic fluorescence of delipidated sEPCR. However, PAF did modify it, suggesting there was an interaction. Interestingly, the affinity was again better than that obtained with PCh (K_D = 16.1 ± 12.0 μM).

To gain further evidence regarding the interaction between sEPCR and lysoPCh or PAF we incubated delipidated sEPCR with lysoPCh (a mixture of 16:0 and 18:0) or PAF, removed the excess of lipid by gel exclusion chromatography and extracted the lipid fraction bound to the purified protein to subject it to mass spectrometry analysis. Peaks were obtained at both *m/z* 496 and 524 in the case of LysoPCh, which correspond to the MW of the lysoPCh molecules used in the experiment. Furthermore, the fragmentation spectrum was identical to that

obtained with pure lysoPCh 16:0 or 18:0. These results confirm that lysoPCh does bind to EPCR (Supplemental Figure 2). In the case of PAF, we detected a peak at *m/z* 524, which corresponds to the molecular weight of PAF, and the fragmentation spectrum was identical to that obtained with pure PAF (Supplemental Figure 3).

LysoPCh and PAF displace PCh from the EPCR pocket and impair APC binding

The finding that these "one-armed" phospholipids bind to EPCR prompted us to determine whether the presence of this lipid could alter EPCR interaction with APC. We incubated delipidated sEPCR with lysoPCh and observed that, unlike PCh, lysoPCh did not restore the ability of delipidated sEPCR to bind to APC (Figure 3B). Finding out whether lysoPCh was able to displace PCh from the EPCR hydrophobic groove thus became a particularly appealing aim. For this reason we incubated sEPCR (note that in this case sEPCR still retains its natural phospholipid) with a 100 molar excess of lysoPCh 16:0, 18:0 or 18:1. Then we captured this sEPCR with RCR-2 mAb immobilized on a SPR sensor chip surface in order to check its ability to bind to APC. The binding was again notably reduced (Figure 3C), which not only provides further evidence that lysoPCh abrogates the EPCR ligand interaction properties but interestingly means that lysoPCh is able to actively displace the original lipid from the hydrophobic pocket.

We also examined the effect of PAF on the sEPCR ability to bind to APC. We demonstrated that the APC binding ability of delipidated sEPCR was not recovered upon PAF reconstitution. The same result i.e. reduced binding, was observed when sEPCR was preincubated with 100 molar excess PAF and subsequently tested with immobilized APC (Figure 3D).

The binding of APC to EPCR on endothelial cells is modulated by the action of $sPLA_2$-V

It was worthwhile at this point to examine whether lysoPCh and PAF were also able to bind to EPCR on the cell surface and thus decrease its APC binding efficiency. For this purpose we incubated HAEC or EA.hy 926 cells with lysoPCh or PAF for 24 and 48 hours. Flow cytometry analyses showed no change in the APC binding capacity (data not shown), suggesting that EPCR on the endothelial surface cannot change its original lipid by exogenously supplied lysoPCh or PAF, as had previously been demonstrated for the homologous CD1d.[17] However, whether *in situ*-produced lysoPCh and PAF were able to interact with cell surface EPCR still remained unanswered. LysoPCh and PAF are originated by the action of $sPLA_2$-V, which is constitutively expressed by endothelial cells and increased by inflammatory stimuli like TNF-α, VEGF and atherosclerosis.[18-20] Therefore, once we had confirmed that $sPLA_2$-V was expressed in HAEC and EA.hy926 (Supplemental Figure 4) we focused on modulating its activity and checking APC binding. Interestingly, the blockade of $sPLA_2$-V activity by its inhibitor manoalide increased APC binding to HAEC (K_D = 71.6 ± 11.1 and 32.7 ± 1.9 nM, p = 0.05, n = 3, in the absence or presence of manoalide, respectively). We used 0.5 μM manoalide which was the lower concentration with the maximun effect (data not shown). Accordingly, the exogenous addition of $sPLA_2$-V decreased APC binding (K_D = 330.5 ± 112.6 nM, n = 3, p = 0.05 vs. control, and p = 0.027 vs. manoalide group) (Figure 4A). We incubated the cells with $sPLA_2$-V for 2 hours because this was the time at which the maximum effect was observed (data not shown). Neither manoalide nor $sPLA_2$-V modified the EPCR expression on the cell surface (Supplemental Figure 5). To rule out the possibility of binding of $sPLA_2$-V to the ligand binding site of EPCR we checked by SPR any possible interaction between them and we did not observe any binding (data not shown). We performed similar experiments using the EA.hy926 cell line and obtained identical results: manoalide significantly enhanced APC binding whereas pretreatment with $sPLA_2$-V significantly decreased it, an effect that was reversed by

manoalide (Supplemental Figures 6 and 7). Therefore, we provide enough evidence to consider that lysoPCh and PAF generated on the cell surface can be accommodated within EPCR and that $sPLA_2$-V is a biological modulator of the EPCR function on the surface of the endothelial cells.

EPCR ability to increase APC generation on endothelial cells is modulated through lipid generation by $sPLA_2$-V

Since $sPLA_2$-V influences the binding of APC to EPCR on endothelial cells, and protein C binds to EPCR similarly to APC i.e. through the Gla domain, we speculated that modulating $sPLA_2$-V activity would influence the EPCR-dependent activation of protein C by thrombin on the endothelial surface. As expected, activation of protein C on HAEC was significantly increased in the presence of manoalide and significantly reduced upon exogenous $sPLA_2$-V supplementation (Figure 4B). Flow cytometry experiments showed that thrombomodulin expression on the surface of the cells was not influenced by modulation of $sPLA_2$-V activity (Supplemental Figure 5). The fact that variations in APC generation were due to differences in the K_m (control: 136.1 ± 24.5 nM; manoalide: 99.6 ± 9.1 nM, p = 0.05 vs. control; $sPLA_2$-V: 526.3 ± 173.7 nM, p = 0.037 vs. control) rather than in the V_{max} (control: 0.070 ± 0.008 nM min^{-1}; manoalide: 0.078 ± 0.003 nM min^{-1}; $sPLA_2$-V: 0.068 ± 0.007 nM min^{-1}) conforms with an impairment of protein C binding to EPCR in the presence of $sPLA_2$-V. We obtained similar results using EA.hy926 cells and were able to reverse the effect of $sPLA_2$-V through manoalide supplementation (Supplemental Figures 6 and 7). Thus, the anticoagulant activity of EPCR on endothelial cells can be modulated by $sPLA_2$-V.

EPCR-dependent antiapoptotic effect of APC on endothelial cells is modulated through lipid generation by sPLA-$_2$V

APC is known to exert numerous cellular effects through EPCR binding. Among them, the prevention of the staurosporine-induced apoptosis of endothelial cells has been well characterized. We induced apoptosis in HAEC with staurosporine and observed that the antiapoptotic effect of APC was significantly increased up to 125 ± 15% ($p = 0.037$) upon preincubation with manoalide (Figure 4C). Accordingly, supplementation with exogenous $sPLA_2$-V significantly decreased the antiapoptotic effect of APC down to 46.6 ± 20.7% ($p = 0.037$) (Figure 4C). Under the conditions used in the experiments, $sPLA_2$-V neither increased the apoptosis of the cells nor decreased PAR-1 expression (Supplemental Figure 8). Similar results were also obtained with EA.hy926 cells (Supplemental Figure 7). These results suggest that the inhibitory effects of sPLA2-V go beyond the antithrombotic nature of the protein C/APC-EPCR interaction, as it also reduces its cytoprotective properties on endothelial cells.

$sPLA_2$-V is present in thrombi formed *in vivo*

Keeping in mind the notable effect of $sPLA_2$-V on EPCR function, we speculated that its modulation could play a role in thrombosis. For this reason, we studied the expression of $sPLA_2$-V in the carotid arteries of mice within the area close to the thrombus induced by laser injury. While we detected neither $sPLA_2$-V nor fibrin in the non-injured carotid arteries (Figure 5C, 5D), $sPLA_2$-V expression was abundant in the laser-injured ones: within the thrombi, in the neutrophils trapped, and on the endothelial surfaces where thrombi were attached (Figure 5A, 5B). Interestingly, we also detected positive $sPLA_2$-V staining in a human thrombus formed in the left atrium of a patient with atrial fibrillation as well as on the endocardial surface nearby (Figure 5F, 5G), whereas we detected no staining in the left atrial endocardium of a donor free of cardiac abnormalities (Figure 5E).

DISCUSSION

EPCR improves the efficiency of protein C activation and is involved in cell signaling mechanisms resulting in antiapoptotic, antiinflammatory and endothelial barrier protective effects.[4,9] When the crystal structure of EPCR was solved, a phospholipid inside its hydrophobic groove was found. However, the nature of this phospholipid remained yet to be elucidated.[11] We provide enough evidence to claim that the major lipid located within the hydrophobic pocket of EPCR is PCh. Of note and according to previous studies,[11] its removal caused a remarkable decrease in APC affinity, which was restored upon PCh supplementation. A similar finding was obtained with FVIIa, thus confirming the important role played by the phospholipid in ligand binding.[3] Our results are consistent with those obtained with the highly homologous CD1d, which is also loaded with PCh in its hydrophobic groove. Furthermore, a recent "in silico" simulation suggested a relationship between the phospholipid binding to EPCR and the size of the surface of interaction with protein C, which would be reduced i.e. ligand binding would be worsened, in the absence of the phospholipid.[21]

The key role played by PCh in EPCR function led us to speculate that its substitution by another lipid could influence its ligand binding ability. Lipid exchange occurs in CD1d and was anticipated for EPCR in the "in silico" study.[21] Among bioactive lipids, lysoPCh exhibits a high structural homology with PCh, since it is the product of an enzymatic process that removes one of the fatty acids from the latter. Furthermore, lysoPCh binds to CD1d.[17,22] For these reasons we studied whether EPCR was able to bind to lysoPCh. Among the lysoPCh variants, we choose the most abundant ones in human physiology.[23] When incubated with delipidated EPCR, lysoPCh dose-dependently modified its intrinsic fluorescence. Furthermore, the mass spectrometry pattern of the lipid fraction extracted from the reconstituted EPCR fully corresponded to lysoPCh. Thus, EPCR is able to accommodate

lysoPCh within its hydrophobic groove. Finally, the binding, according to the K_D, was stronger than that observed with PCh and, of note, remarkably impaired the affinity of EPCR for APC and FVIIa (Online Supplemental Discussion). These findings fully conform to the "in silico" simulation.[21] PAF is a bioactive lipid structurally similar to lysoPCh, also generated by endothelial cells, and was able to displace PCh and bind to EPCR as well.

To find out whether lysoPCh and PAF influenced EPCR function in a more physiological condition, we moved to the cellular context. Unlike what happened in the experiments performed with sEPCR, there was no change in the EPCR-dependent APC binding when lysoPCh or PAF were directly added to endothelial cells. This result initially suggested that lipid substitution does not take place when EPCR is on the cell membrane. However, in an attempt to be closer to the *in vivo* condition, we studied whether the EPCR ability to bind to APC was modified upon manipulation of $sPLA_2$-V, an endothelially expressed enzyme which is the main source of lysoPCh and PAF in that tissue:[24] $sPLA_2$-V belongs to the superfamily of PLA2 enzymes[25] and, being constitutively expressed by endothelial cells, it is upregulated by inflammatory stimuli like TNF-α, VEGF or in inflammatory conditions such as atherosclerosis. Firstly, we incubated endothelial cells with the PLA2 inhibitor manoalide prior to assess APC binding. Unlike what happened when lysoPCh was directly given to cells, manoalide did increase the EPCR-dependent binding of APC, and this had to be due to the inhibition of $sPLA_2$-V: although manoalide also inhibits $sPLA_2$-IIa and X, the former is not present in resting endothelial cells[18-20] and the latter is not present either, as we showed (Online Figure II). Accordingly, APC binding was notably reduced when endothelial cells were supplemented with $sPLA_2$-V. Since SPR experiments made us discard a direct interaction between EPCR and $sPLA_2$-V, the most likely explanation for these results is that $sPLA_2$-V generates lysoPCh and PAF in the vicinity of EPCR, and the lipids so generated displace PCh from EPCR, which in turn partially loses its binding capacity. The fact that a

similar mechanism has recently been demonstrated for the highly homologous CD1d reinforces our hypothesis.[17]

As anticipated, the changes in the functionality of EPCR through $sPLA_2$-V had relevant biological consequences i.e. the activation of protein C by thrombin and the antiapoptotic effect of APC were impaired as the $sPLA_2$-V activity was increased and viceversa. Thus, a new role for $sPLA_2$-V as a downregulator of EPCR function can be proposed, which would be specially important in scenarios of inflammation, in which its endothelial expression is enhanced. Since APC is one of the cornerstones of the crosstalk between inflammation and coagulation, it is interesting that we detected $sPLA_2$-V in the endothelium in situations of injury leading to thrombosis, i.e. murine carotid artery, while it was not detected in non-injured vessels. Furthermore, not only was $sPLA_2$-V detected on the endothelium but also inside the thrombus and on the trapped neutrophils, which suggests that $sPLA_2$-V activity was increased upon insult, and in turn the locally generated lysoPCh and PAF diminished the protein C/APC binding ability of EPCR thus exacerbating the coagulative and inflammatory responses in the vicinity of the injured area, thereby facilitating thrombus formation. This theory is further substantiated by previous literature supporting a role for thrombin as a trigger of the release of lysoPCh by endothelial cells which could be mediated by an increase in $sPLA_2$-V expression.[26] Furthermore, detection of $sPLA_2$-V in neutrophils and release upon cell activation have also been previously reported.[27] Finally, the presence of $sPLA_2$-V in the left atrial wall, with an attached thrombus, of a patient with atrial fibrillation, suggests that our observations can be applied to humans.

Our findings can be clinically relevant. Overexpression of $sPLA_2$-V would not only reduce the amount of APC generated but would also impair the APC-dependent cell signaling through the EPCR-PAR-1 axis, thus reducing the APC-associated benefit in a variety of settings as endothelial barrier protection, apoptosis reduction, metastasis prevention and

others. Also, circulating factor VII levels could become higher since a role for EPCR as a scavenger for factor VII, that would be cleared from the circulation, has been demonstrated.[28] Moreover, since we demonstrated that EPCR decreases the activation of FVII by factor Xa,[5] when $sPLA_2$-V was overexpressed, FVIIa generation would be increased thus reinforcing the procoagulant properties that our results confer to sPLA2-V.

In summary, we have demonstrated that the major lipid bound to the hydrophobic groove of EPCR is PCh. PCh can be replaced by $sPLA_2$-V-produced lysoPCh or PAF. As a consequence, the affinity of EPCR for protein C/APC and factor VII/VIIa decreases, which leads to a reduction in the endothelial capacity to generate APC and in the APC-evoked cell protective mechanisms. A new prothrombotic/proinflammatory role can be attributed to $sPLA_2$-V, which emerges as a promising therapeutic target. Its modulation may help to take greater advantage of the anticoagulant and cytoprotective actions of the APC-EPCR axis.

ACKNOWLEDGEMENTS

We thank Eva Molina and Maider Esparza for their excellent technical assistance. We thank also Fernando Corrales and Carmen Miqueo from the Proteomics facility of CIMA, a member of ProteoRed-ISCIII, for their assistance performing mass spectrometry experiments. This work was supported through the Unión Temporal de Empresas project CIMA and by grants from Instituto de Salud Carlos III (PI08/1349, PI10/01432 and Red Temática de Investigación RECAVA RD06/0014/0008) and Health Department, Gobierno de Navarra (15/09). Jacinto López-Sagaseta was supported by a fellowship from the Education Department, Gobierno de Navarra.Jorge Cerveró was supported by a grant from the Sociedad Española de Trombosis y Hemostasia SETH/FETH.

AUTHORSHIP CONTRIBUTION

JL-S, CP, RM and JH designed research. Jl-S, CP, MA, JC and SEV performed research, CTE contributed vital new reagents. JL-S, CP, CTE, RM and JH analyzed and interpreted data. JL-S, CP, RM and JH wrote the manuscript.

DISCLOSURES

None.

REFERENCES

1. Laszik Z, Mitro A, Taylor FB, Jr., Ferrell G, Esmon CT. Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. Circulation. 1997;96:3633-3640.
2. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. J Biol Chem. 1994;269:26486-26491.
3. Lopez-Sagaseta J, Montes R, Puy C, Diez N, Fukudome K, Hermida J. Binding of factor VIIa to the endothelial cell protein C receptor reduces its coagulant activity. J Thromb Haemost. 2007;5:1817-1824.
4. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci U S A. 1996;93:10212-10216.
5. Puy C, Lopez-Sagaseta J, Hermida J, Montes R. The endothelial cells downregulate the generation of factor VIIa through EPCR binding. Br J Haematol. 2010;149:111-117.
6. Koster T, Rosendaal FR, Briet E, et al. Protein C deficiency in a controlled series of unselected outpatients: an infrequent but clear risk factor for venous thrombosis (Leiden Thrombophilia Study). Blood. 1995;85:2756-2761.
7. Zorio E, Navarro S, Medina P, et al. Circulating activated protein C is reduced in young survivors of myocardial infarction and inversely correlates with the severity of coronary lesions. J Thromb Haemost. 2006;4:1530-1536.
8. Folsom AR, Ohira T, Yamagishi K, Cushman M. Low protein C and incidence of ischemic stroke and coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) Study. J Thromb Haemost. 2009;7:1774-1778.
9. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood. 2007;109:3161-3172.
10. Bezuhly M, Cullen R, Esmon CT, et al. Role of activated protein C and its receptor in inhibition of tumor metastasis. Blood. 2009;113:3371-3374.
11. Oganesyan V, Oganesyan N, Terzyan S, et al. The crystal structure of the endothelial protein C receptor and a bound phospholipid. J Biol Chem. 2002;277:24851-24854.
12. De Libero G, Collmann A, Mori L. The cellular and biochemical rules of lipid antigen presentation. Eur J Immunol. 2009;39:2648-2656.
13. Hurtado V, Montes R, Gris JC, et al. Autoantibodies against EPCR are found in antiphospholipid syndrome and are a risk factor for fetal death. Blood. 2004;104:1369-1374.
14. Lopez-Sagaseta J, Montes R, Hermida J. Recombinant expression of biologically active murine soluble EPCR. Protein Expr Purif. 2009;64:194-197.

15. Fukudome K, Kurosawa S, Stearns-Kurosawa DJ, He X, Rezaie AR, Esmon CT. The endothelial cell protein C receptor. Cell surface expression and direct ligand binding by the soluble receptor. J Biol Chem. 1996;271:17491-17498.
16. Centelles MN, Puy C, Lopez-Sagaseta J, Fukudome K, Montes R, Hermida J. Blocking endothelial protein C receptor (EPCR) accelerates thrombus development in vivo. Thromb Haemost. 2010;103:1239-1244.
17. Fox LM, Cox DG, Lockridge JL, et al. Recognition of lyso-phospholipids by human natural killer T lymphocytes. PLoS Biol. 2009;7:e1000228.
18. Sonoki K, Iwase M, Sasaki N, et al. Secretory PLA2 inhibitor indoxam suppresses LDL modification and associated inflammatory responses in TNFalpha-stimulated human endothelial cells. Br J Pharmacol. 2008;153:1399-1408.
19. Bernatchez PN, Winstead MV, Dennis EA, Sirois MG. VEGF stimulation of endothelial cell PAF synthesis is mediated by group V 14 kDa secretory phospholipase A2. Br J Pharmacol. 2001;134:197-205.
20. Rosengren B, Peilot H, Umaerus M, et al. Secretory phospholipase A2 group V: lesion distribution, activation by arterial proteoglycans, and induction in aorta by a Western diet. Arterioscler Thromb Vasc Biol. 2006;26:1579-1585.
21. Chiappori F, Merelli I, Milanesi L, Rovida E. Exploring the role of the phospholipid ligand in endothelial protein C receptor: a molecular dynamics study. Proteins. 2010;78:2679-2690.
22. Cox D, Fox L, Tian R, et al. Determination of cellular lipids bound to human CD1d molecules. PLoS One. 2009;4:e5325.
23. Drobnik W, Liebisch G, Audebert FX, et al. Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. J Lipid Res. 2003;44:754-761.
24. Han SK, Kim KP, Koduri R, et al. Roles of Trp31 in high membrane binding and proinflammatory activity of human group V phospholipase A2. J Biol Chem. 1999;274:11881-11888.
25. Burke JE, Dennis EA. Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res. 2009;50 Suppl:S237-242.
26. McHowat J, Corr PB. Thrombin-induced release of lysophosphatidylcholine from endothelial cells. J Biol Chem. 1993;268:15605-15610.
27. Degousee N, Ghomashchi F, Stefanski E, et al. Groups IV, V, and X phospholipases A2s in human neutrophils: role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. J Biol Chem. 2002;277:5061-5073.
28. Nayak RC, Sen P, Ghosh S, et al. Endothelial cell protein C receptor cellular localization and trafficking: potential functional implications. Blood. 2009;114:1974-1986.

FIGURE LEGENDS

Figure 1. Identification of the phospholipid bound to EPCR. (A) Lane 1, a mixture of PE and PCh was loaded as standards. Lane 2, lipid fraction extracted from yeast-produced sEPCR. Lane 3, lipid fraction extracted from yeast-produced murine sEPCR. Lane 4, lipid fraction from sEPCR produced in a mammalian expression system. (B) MS/MS fragmentation analysis of the lipid fraction from yeast-produced sEPCR. The peak at m/z 786 matches up with commercial PCh. The spectrum generated by fragmentation of the molecule at m/z 786 fits properly with commercial PCh. An inset with the different PCh polar group fragmentation products and their respective m/z is included.

Figure 2. SPR analysis of the influence of PCh on the APC binding to sEPCR. sEPCR was captured on a CM5 chip through RCR-2 mAb. (A) Binding of APC (0, 3, 9, 27, 81 and 243 nM) to sEPCR. (B) Binding of APC (0, 9, 27, 81, 243 and 729 nM) to delipidated sEPCR. In both cases a representative experiment out of three independent repeats is shown. Black lines represent experimental data, grey lines represent fittings to a Langmuir 1:1 kinetic model. (C) Binding at equilibrium of 100 nM APC to sEPCR, delipidated sEPCR and delipidated sEPCR reconstituted with 760 and 786 Da MW PCh. The mean ± standard deviation (SD) of three independent experiments are represented. Mann-Whitney U-test was used for statistical comparisons.

Figure 3. Binding of lysoPCh to sEPCR. (A) Delipidated sEPCR was incubated with increasing amounts of 16:0, 18:0 and 18:1 lysoPCh, 760 Da MW PCh and PAF and the change in the sEPCR intrinsic fluorescence was registered. A representative experiment is showed for each lipid. (B) SPR analysis of the binding of APC to delipidated sEPCR

reconstituted with PCh or lysoPCh. sEPCR or delipidated sEPCR were captured on a CM5 chip through RCR-2 mAb. Binding at equilibrium of 100 nM APC to sEPCR and to delipidated sEPCR preincubated or not with PCh or lysoPCh is shown. The mean±SD of three independent experiments are represented. Mann-Whitney U-test was used for statistical comparisons. Dlip-sEPCR, delipidated sEPCR. (C) SPR analysis of the binding of APC to sEPCR reconstituted with lysoPCh. sEPCR was captured on a CM5 chip through RCR-2 mAb. Binding at equilibrium of 100 nM APC to sEPCR preincubated or not with different species of lysoPCh is shown. The mean ± SD of three independent experiments are represented. Mann-Whitney U-test was used for statistical comparisons. (D) SPR analysis of the binding of APC to sEPCR reconstituted with PAF. APC-PPACK-b was captured onto a SA chip. Binding at equilibrium of 100 nM sEPCR, delipidated sEPCR, PAF-relipidated sEPCR and sEPCR preincubated with PAF are shown. The mean±SD of three independent experiments are shown. Mann-Whitney U-test was used for statistical comparisons. Dlip-sEPCR, delipidated sEPCR.

Figure 4. Effect of $sPLA_2$-V on EPCR function on endothelial cells. (A) APC binding to HAEC. Cells were incubated with increasing amounts of APC-PP* after 48-hour-pretreatment with 0.5 µM manoalide (○), 2 hours-pretreatment with 20 µg/mL sPLA2-V (●) or no pretreatment (●). APC-PP* binding was assessed by flow cytometry. A representative experiment out of three independent repeats is shown. MEFL means molecules of equivalent fluorescein. (B) Protein C activation on HAEC. Increasing amounts of protein C were incubated with thrombin for 30 minutes (min) in the presence of HAEC, 48 hours-pretreated with 0.5 µM manoalide (○), 2 hours-pretreated with 20 µg/mL sPLA2-V (●), or non-pretreated (●). The amount of APC generated was measured with the chromogenic substrate S-2366. A representative experiment out of three independent repeats is shown. (C)

Inhibitory effect of APC on staurosporine-induced apoptosis in HAEC. Cells were pretreated with manoalide or sPLA2-V as in A and B, and then supplemented with 50 nM APC for 4 hours after which apoptosis was induced with 10 µM staurosporine for 60 min. Apoptosis was estimated by assessing the number of cells positive for annexin V-Alexa 647 binding by flow cytometry. The antiapoptotic effect of APC in the absence of manoalide and sPLA2-V was considered 100%. The mean ± SD of three independent experiments are shown. Mann-Whitney U-test was used for statistical comparisons.

Figure 5. Immunohistochemical detection of sPLA2-V in sections of injured vessels with an attached thrombus. (A-D) Carotid artery sections of a mouse subjected to left carotid artery laser injury model. Rabbit anti-mouse sPLA2-V Ab (A and C) or Masson's Trichrome for fibrin detection (B and D) were used. (A) sPLA2-V was present in the injured carotid artery: in endothelial cells (arrow), neutrophils (arrowhead), vascular smooth muscle cells (asterisk) and around fibrin in the lumen. (B) The soft purple staining in the lumen of the injured carotid artery revealed the presence of extensive fibrin deposits. (C) There was no sPLA2-V in the non-injured carotid artery. (D) Fibrin was not detected in the non-injured artery. (E-G) The presence of sPLA2-V was also studied in human left atrial tissue sections. (E) sPLA2-V was absent in the atrial sample of a patient in sinus rhythm. (F-G) sPLA2-V was detected around the fibrin thrombus and along the endothelial lining in the atrial section corresponding to a patient in atrial fibrillation

FIGURE 1

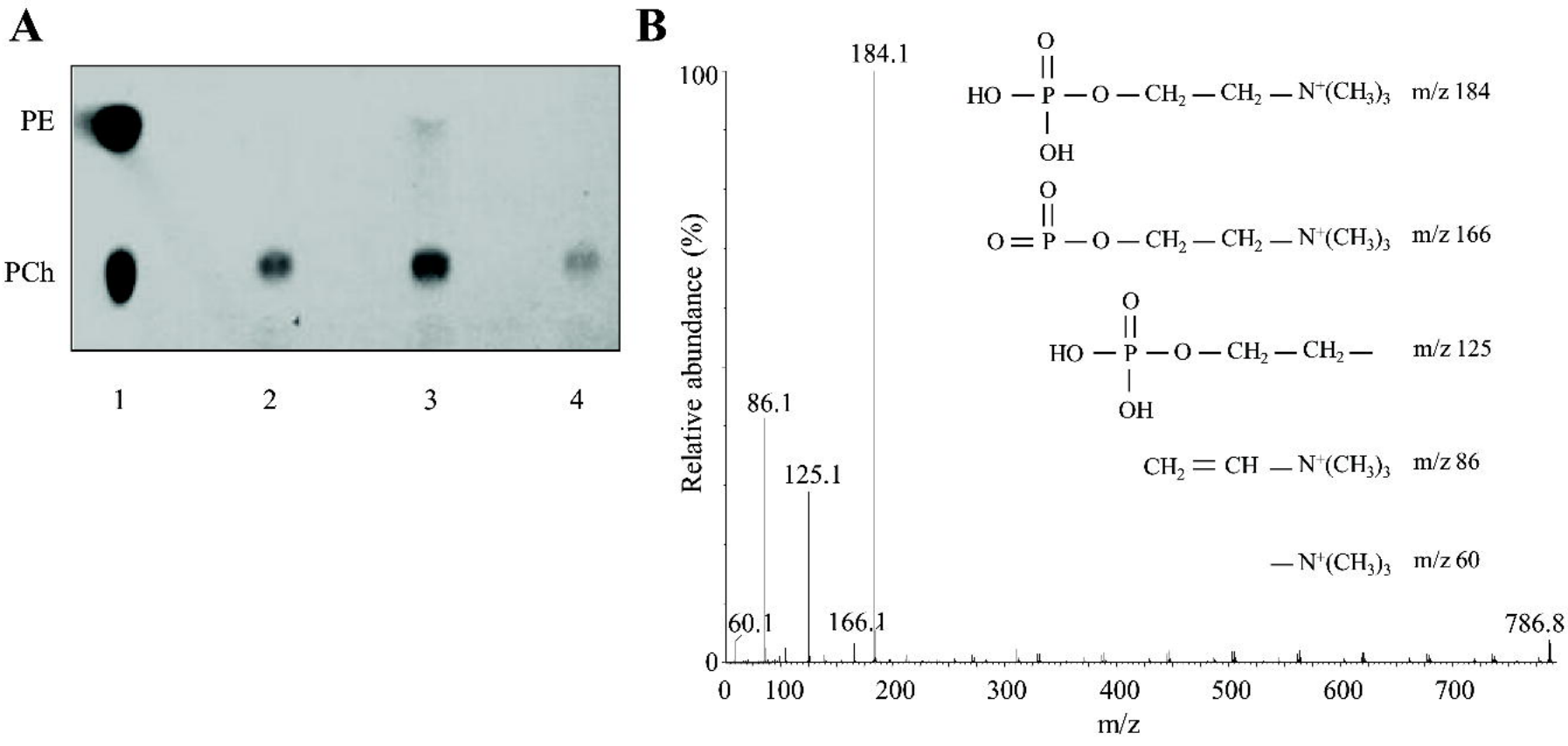
A
PE
PCh
1
2
3
4
B
184.1
100
Relative abundance (%)
86.1
125.1
60.1
166.1
786.8
0
0
100
200
300
400
500
600
700
m/z
m/z 184
m/z 166
m/z 125
m/z 86
m/z 60

FIGURE 2

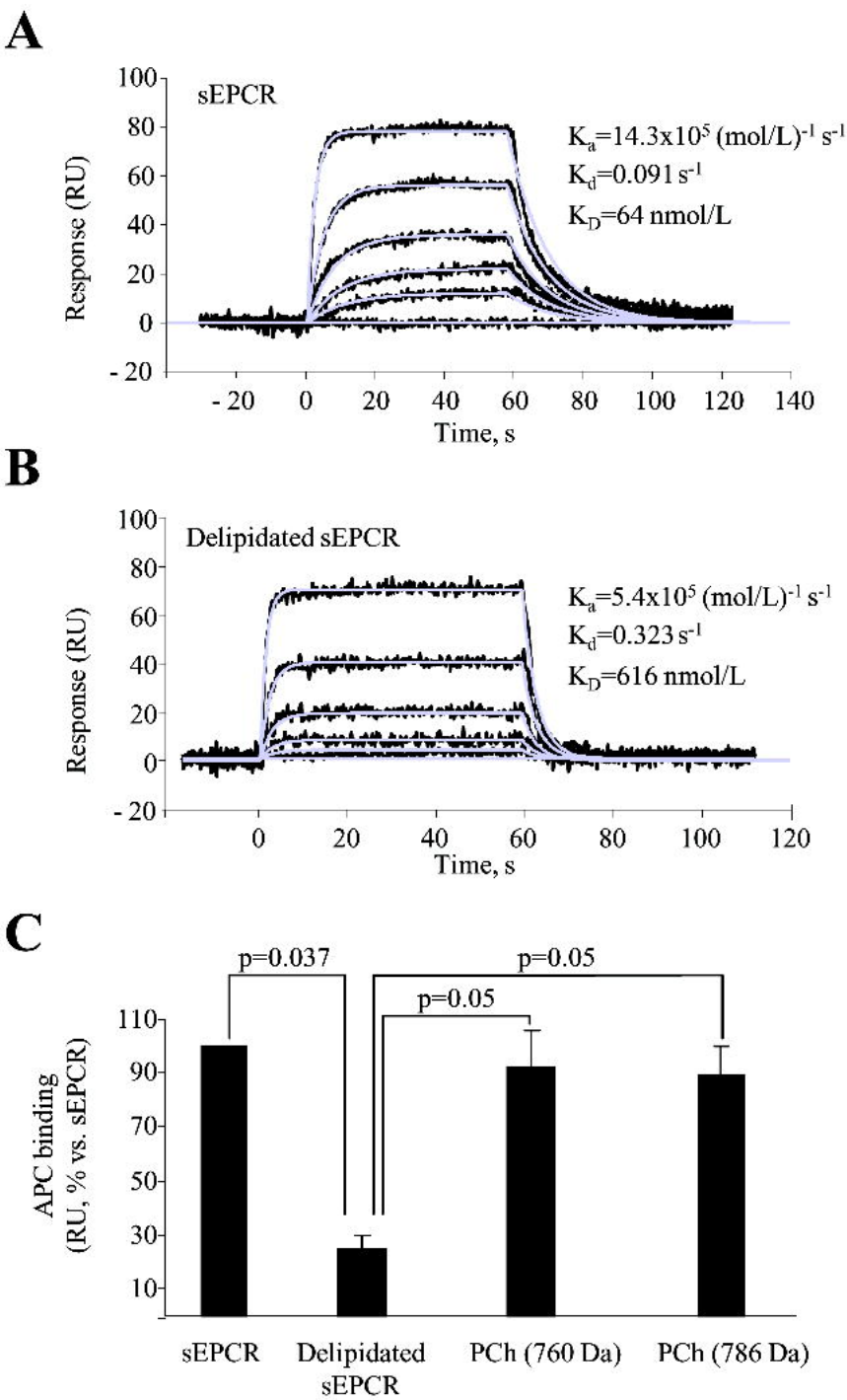
A
sEPCR
K_a=14.3x10^5 (mol/L)$^{-1}$ s^{-1}
K_d=0.091 s^{-1}
K_D=64 nmol/L
Response (RU)
Time, s
B
Delipidated sEPCR
K_a=5.4x10^5 (mol/L)$^{-1}$ s^{-1}
K_d=0.323 s^{-1}
K_D=616 nmol/L
Response (RU)
Time, s
C
p=0.037
p=0.05
p=0.05
APC binding (RU, % vs. sEPCR)
sEPCR
Delipidated sEPCR
PCh (760 Da)
PCh (786 Da)

FIGURE 3

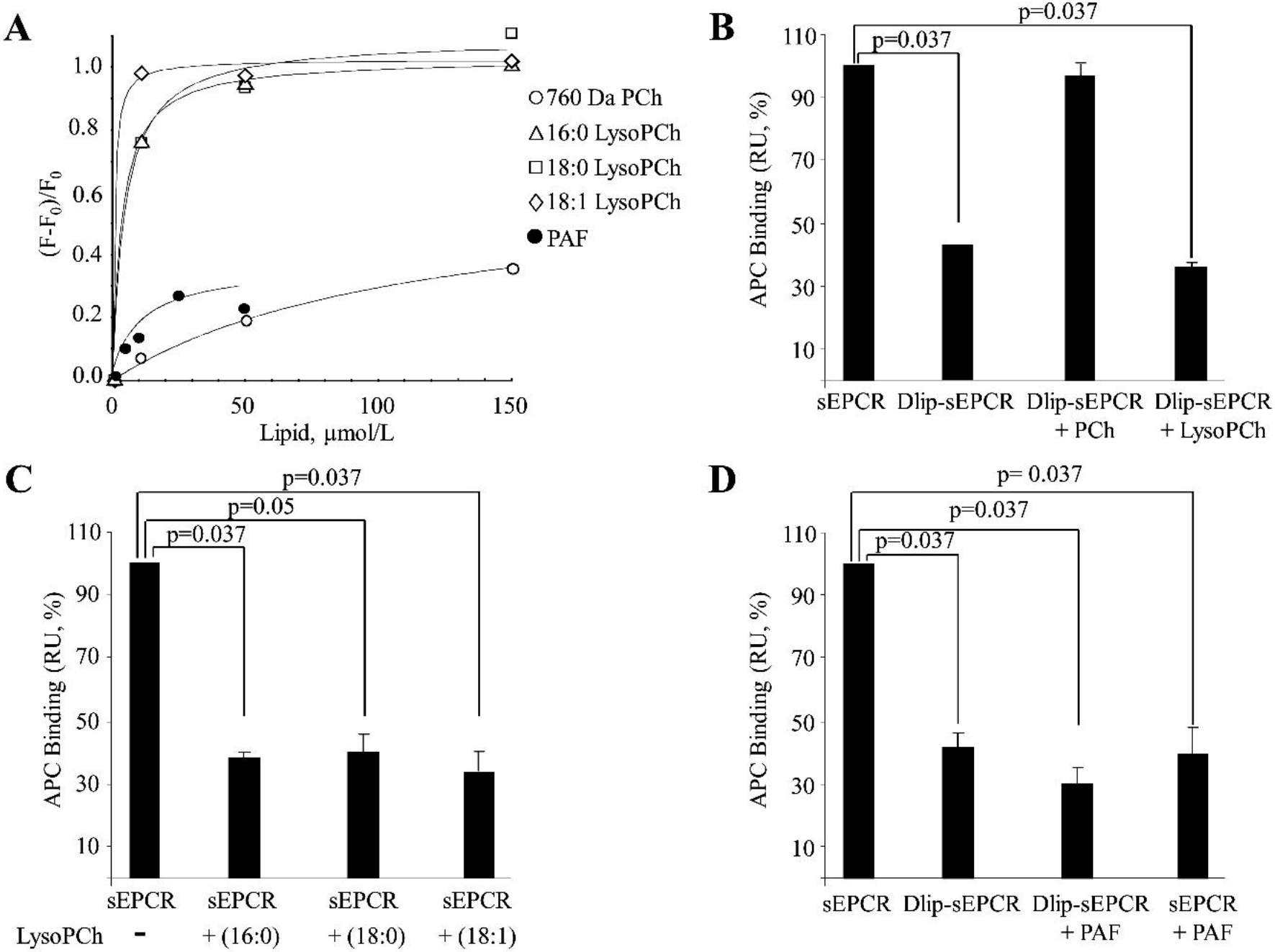
A
1.0
0.8
0.6
0.4
0.2
0.0
(F-F0)/F0
0
50
100
150
Lipid, µmol/L
760 Da PCh
16:0 LysoPCh
18:0 LysoPCh
18:1 LysoPCh
PAF
B
p=0.037
p=0.037
110
90
70
50
30
10
APC Binding (RU, %)
sEPCR
Dlip-sEPCR
Dlip-sEPCR + PCh
Dlip-sEPCR + LysoPCh
C
p=0.037
p=0.05
p=0.037
110
90
70
50
30
10
APC Binding (RU, %)
sEPCR
sEPCR + (16:0)
sEPCR + (18:0)
sEPCR + (18:1)
LysoPCh -
D
p= 0.037
p=0.037
p=0.037
110
90
70
50
30
10
APC Binding (RU, %)
sEPCR
Dlip-sEPCR
Dlip-sEPCR + PAF
sEPCR + PAF

FIGURE 4

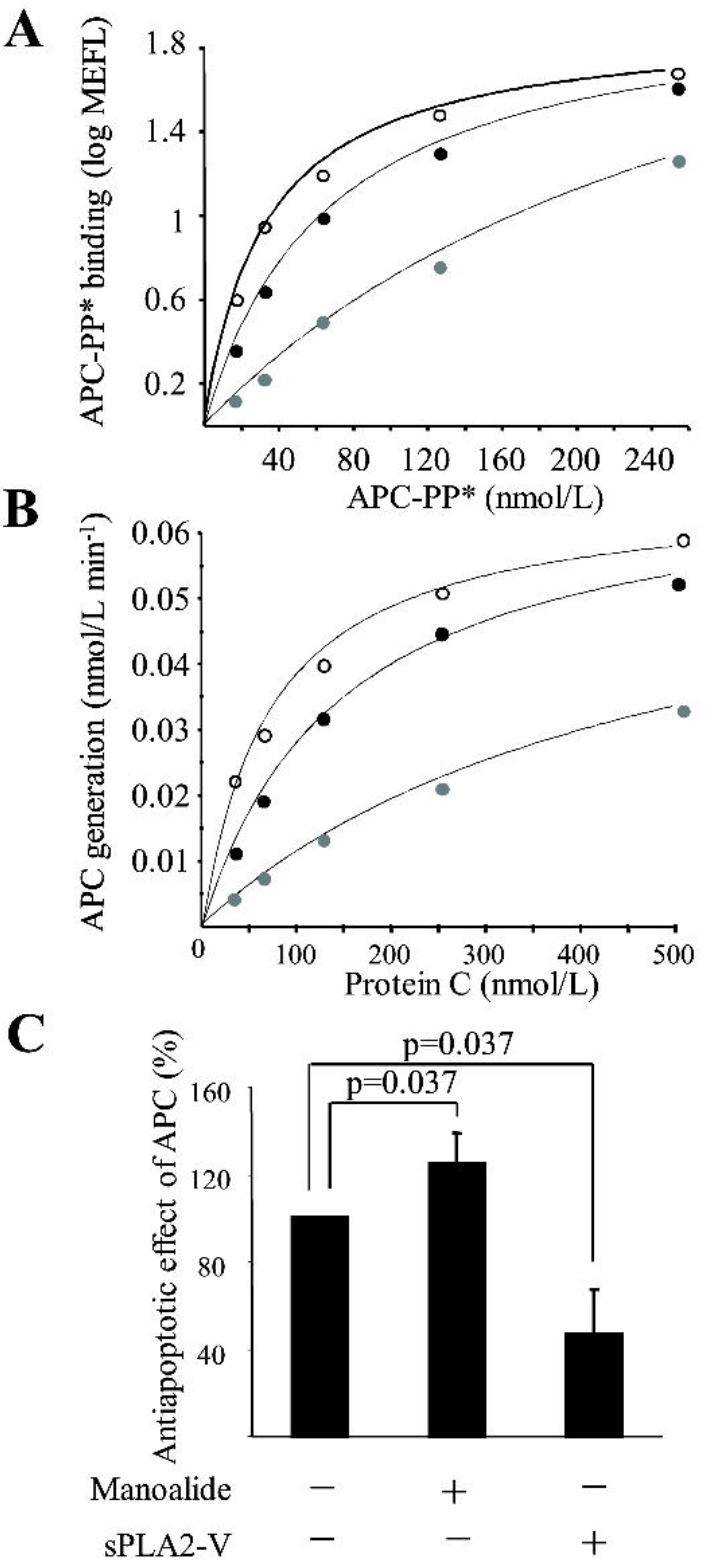
A
APC-PP* binding (log MEFL)
1.8
1.4
1
0.6
0.2
40
80
120
160
200
240
APC-PP* (nmol/L)
B
APC generation (nmol/L min-1)
0.06
0.05
0.04
0.03
0.02
0.01
0
100
200
300
400
500
Protein C (nmol/L)
C
Antiapoptotic effect of APC (%)
p=0.037
p=0.037
160
120
80
40
Manoalide
−
+
−
sPLA2-V
−
−
+

FIGURE 5