Regulation of the antithrombotic activity of the endothelial cell protein C receptor by bioactive lipids

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EPCR is at the center of the protein C/activated protein C-dependent mechanisms

The endothelial cell protein C/activated protein C (APC) receptor (EPCR) is a transmembrane glycoprotein which binds to protein C/ APC and factor VII/active VII (FVIIa) with high affinity [dissociation constant (Kd) ≈ 30-70 nM][1,2]. EPCR is found in the endothelium, most abundantly in large arterial vessels. EPCR has also been detected in other cells like trophoblasts, vascular smooth muscle cells (VSMC), immune system cells, etc., although its relevance in these settings has been less widely studied[3].

EPCR plays an important role in haemostasis by facilitating the activation of protein C by thrombin bound to the endothelial receptor thrombomodulin. EPCR lowers the Michaelis-Menten constant (Km) of the protein C activation more than 10-fold[4]. In vivo, EPCR increases the APC generation by around ten-fold[5]. The fact that low APC levels are associated with a higher thrombotic risk[6] and that blockade of EPCR accelerates vessel thrombotic occlusion emphasizes the role played by EPCR in preventing clot formation[7].

On the other hand, EPCR is required for APC to start cell signalling mechanisms. Through binding to the receptor, APC activates the protease activated receptor-1 (PAR-1), thus triggering a variety of signal transduction pathways which result in antiadhesive and antiapoptotic patterns, and in the reinforcement of the endothelial barrier[8]. EPCR also facilitates the transport of APC through the blood brain barrier[9]. In fact, APC may play a promising role in ischemic stroke treatment, as suggested by previous animal work[10]. A clinical trial examining the usefulness of exogenously administered APC in this pathology is being carried out (NCT00533546; http://www.clinicaltrials.gov). Furthermore, the bleeding side effects associated with APC therapy will most probably be overcome with the use of APC mutants able to bind to EPCR but unable to inactivate factors V and VIII[11,10].

Therefore, a fully functional EPCR on the endothelial surface is mandatory to ensure proper protein C activation and APC-dependent cell signalling. Regulation of EPCR has been poorly studied. It is known that thrombin, tumour necrosis factor-α and other inflammatory mediators may influence receptor levels and shedding[11,12], but, until recently, nothing was known about possible post-translational mechanisms.

The influence of the phospholipid in the ligand binding ability of EPCR

Once the crystal structure of the extracellular region of EPCR was resolved, it was shown that a phospholipid was located within a hydrophobic groove formed upon folding of the molecule into two alpha chains and a β-sheet platform. Although the phospholipid had to be phosphatidylcholine (PC) or phosphatidylethanolamine, its exact nature could not be identified. However, it was concluded that its presence was necessary to preserve the ligand binding properties of EPCR[12].

The powerful system set up in our laboratory to produce recombinant soluble EPCR (sEPCR) in yeast[13] permitted us to perform the experiments required to identify this phospholipid. Thin layer chromatography and mass spectrometry approaches allowed us to state that PCh is the phospholipid present within the EPCR groove. Confirmatory experiments with mammalian cell-produced sEPCR and with sEPCR spontaneously shed from human endothelial cells yielded the same result. Furthermore, the removal of PCh notably impaired the ligand binding ability of EPCR[14].

The similarity in size and structure between PCh and some bioactive “one-armed” phospholipids, i.e. lysophosphatidylcholine (lysoPCh) and platelet activating factor (PAF), known to be present in inflammatory conditions[15], is remarkable (Figure 1). This background led us to hypothesize that the EPCR hydrophobic groove would be able to allocate one of these molecules instead of PCh, and that, as a consequence, the ligand binding ability of EPCR would be compromised. Provided that this was true, the protein C/APC-dependent mechanisms (i.e. anticoagulation/cell signaling) would not work properly in situations where high amounts of lysoPCh or PAF were in the vicinity of the endothelial surface.

EPCR is able to allocate bioactive lipids within its hydrophobic groove

Taking advantage of the detectable change in the intrinsic fluorescence of a molecule upon structural modification, the ability of lysoPCh and PAF to reconstitute previously delipidated sEPCR was tested. Both lipids interacted with delipidated sEPCR. Furthermore, the Kd that governed such reactions was between 6 and 15-fold lower than that obtained upon sEPCR reconstitution with PCh[14]. A question emerged: would EPCR be able to preserve its ligand binding ability when lysoPCh or PAF were allocated within its groove instead of PCh? The surface plasmon resonance (SPR) approach showed us that its function was severely compromised upon replacement of PCh with lysoPCh or PAF (Figure 2): its APC binding ability, which was severely decreased to around 50% when it had previously been delipidated, did not recover upon lysoPCh or PAF reconstitution, while it did recover upon PCh reconstitution. Even more, incubation of fully functional, PCh-bearing sEPCR with lysoPCh or PAF also ruined the binding ability of the receptor. Mass spectrometry analyses showed...
LysoPCh and PAF originate through the action of sPLA2-V on PCh. sPLA2-V is constitutively expressed by endothelial cells and can be found in the circulation and bound to the outer leaflet of the plasma membrane as well. Its expression can be increased by a variety of inflammatory stimuli(16). We hypothesized that a gain-of-function in sPLA2-V activity on the endothelial surface would impair EPCR function and vice versa. To test this notion, we first incubated sPLA2-V with sEPCR and observed by SPR that, as a result, the APC binding ability of the latter was substantially impaired(14). Subsequently, to study whether this finding could also be observed in a more physiological setting, we used human aortic endothelial cells (HAEC), which constitutively express sPLA2-V. HAEC were incubated with the phospholipase inhibitor mannoalide, or supplemented with sPLA2-V to down or up-regulate sPLA2-V activity, respectively, in the vicinity of EPCR. Several end-points were subsequently approached to evaluate EPCR function on the HAEC surface: extent of APC binding, protein C activation and APC-dependent prevention of apoptosis. Figure 3 summarizes these experiments. In the presence of mannoalide, EPCR function was improved, as demonstrated by: a superior ability of the cells to bind APC, which is fully EPCR-dependent; an increased activation of protein C on the endothelial surface upon thrombin supplementation, which is highly dependent on EPCR; an increased antiapoptotic effect of APC on staurosporine-treated cells, which is fully EPCR-dependent. Accordingly, supplementation with sPLA2-V exerted the opposite effect. To discard the possibility that the effect of mannoalide was due to the inhibition of phospholipases other than sPLA2-V, additional experiments to specifically silence the sPLA2-V gene were performed. sPLA2-V silencing resulted in a two-fold increase in APC generation when the ability of such cells to sustain protein C activation was tested, confirming that sPLA2-V activity does influence EPCR function(14).

Taking these data collectively, what we learn is that EPCR activity can be regulated beyond the level of transcription, and that sPLA2-V plays a so far unsuspected role in the control of the protein C/APC-related mechanisms, thus becoming a new actor in the crosstalk between haemostasis and inflammation.

sPLA2-V is present in thrombi and at sites of endothelial injury

All the experimental data presented so far were obtained in purified systems or cell cultures. Once the ability of sPLA2-V to impair EPCR

that the bioactive lipids had replaced PCh(14). Thus, lipid exchange is also possible without the previously induced delipidation.

All these arguments invited us to think that allocation of lysoPCh or PAF, instead of PCh, within the hydrophobic groove of EPCR, could be physiologically relevant and that high lysoPCh or PAF levels in the vicinity of EPCR should facilitate the process. This led us to follow the trace of the mechanisms that can provide the endothelial surface with these lipids. As a consequence, we focused on the secretory group V phospholipase A2 (sPLA2-V).

Modulation of the sPLA2-V activity influences EPCR function on the endothelial surface

LysoPCh and PAF bind APC, which is fully EPCR-dependent; an increased activation of protein C on the endothelial surface upon thrombin supplementation, which is highly dependent on EPCR; an increased antiapoptotic effect of APC on staurosporine-treated cells, which is fully EPCR-dependent. Accordingly, supplementation with sPLA2-V exerted the opposite effect. To discard the possibility that the effect of mannoalide was due to the inhibition of phospholipases other than sPLA2-V, additional experiments to specifically silence the sPLA2-V gene were performed. sPLA2-V silencing resulted in a two-fold increase in APC generation when the ability of such cells to sustain protein C activation was tested, confirming that sPLA2-V activity does influence EPCR function(14).

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function had been categorically demonstrated, the next step was to look for evidence to support its role in vivo. The easiest way to start was to look for sPLA2-V at sites where clot formation was actively taking place. Endothelial injury induced in mice by laser incidence on the carotid artery is a widely used thrombus formation model. Figure 4A and B show that, while sPLA2-V was not detected in the non-injured vessel, it could be localized within the thrombus and, remarkably, in the neutrophils trapped, and on the endothelial surface where the thrombus was attached. Most interestingly, sPLA2-V was also detected attached to the prothrombotic surface of the left atrial endocardium of a patient with atrial fibrillation, close to a thrombus where sPLA2-V was also present (Figure 4D and E). No traces of sPLA2-V were observed in the left atrial endocardium of a donor free of cardiac abnormalities predisposing to a hypercoagulable state.

Figure 3. Effect of sPLA2-V on EPCR function on endothelial cells. (A) APC binding to HAEC. Cells were incubated with increasing amounts of fluorescently labelled APC after 48-hour pretreatment with 0.5 µM manoalide (●), 2-hour pretreatment with 20 µg/mL sPLA2-V (○), or no pretreatment (●). APC binding was assessed by flow cytometry. A representative experiment out of three independent repeats is shown. APC*, fluorescently labelled APC (biotinylated PPACK-APC bound to Alexa 647-streptavidin); MEFL means molecules of equivalent fluorochrome. (B) Protein C activation on HAEC. Increasing amounts of protein C were incubated with thrombin for 30 minutes with HAEC which had been pretreated for 48 hours with 0.5 µM manoalide (●), pretreated for 2 hours 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. Min, minutes. (C) Inhibitory effect of APC on stauorosporeine-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. Apoptosis was subsequently induced with 10 µM staurosporine for 60 minutes. Apoptosis was estimated by flow cytometry, assessing the number of cells positive for annexin V-Alexa 647 binding. 6.6 ± 1.1% of the untreated cells were apoptotic, and this percentage increased to 15.1 ± 1.0% after staurosporine incubation. Results refer to the untreated cells, whose apoptotic rate was considered 100%. The mean ± SD of three independent experiments is shown. Mann-Whitney U-test was used for statistical comparisons. Sta, staurosporine, *p < 0.05 vs. [staurosorpine + APC] group. This research was originally published in Blood. López-Sagaseta et al. sPLA2-V inhibits EPCR anticoagulant and antiapoptotic properties by accommodating lysophosphatidylcholine or PAF in the hydrophobic groove. Blood 2012;119:2914-21. © the American Society of Hematology.

Conclusions and future directions

The results presented herein demonstrate that the nature of the phospholipid residing within the hydrophobic groove of EPCR strongly influences the function of the latter. LysoPCh and PAF are able to actively displace PCh from the EPCR molecule, thus notably impairing its ability to bind to protein C or APC. Such lipids arise as a consequence of the activity of a phospholipase, sPLA2-V, whose modulation influences the EPCR-dependent functions of protein C and APC in vitro. sPLA2-V has been detected at sites of high thrombogenic activity.

Ongoing research is currently being conducted to demonstrate that EPCR function is influenced by the sPLA2-V activity in vivo. If this is so, there are a few interesting topics to be addressed. How is sPLA2-V modulated in endothelial cells? It is known that its expression is increased in inflammatory conditions like atherosclerosis or endotoxaemia. Furthermore, other cells in the vicinity of endothelium are able to produce and secrete sPLA2-V. We detected sPLA2-V in neutrophils trapped within thrombi; in the prothrombotic environment, is there a stimulus that triggers its expression in those
Figure 4. sPLA2-V is present in sections of injured vessels in mice and men. A and B) Carotid artery sections of a non-injured mouse and a mouse subjected to carotid artery laser injury model respectively. There was no sPLA2-V in the carotid artery of the non-injured mouse. However, sPLA2-V could be observed in the injured carotid artery in endothelial cells (arrow), neutrophils (arrowhead), VSMC (asterisk) and around fibrin in the lumen. (C-E) Human left atrial tissue sections of (C) a patient in sinus rhythm and (D and E) a patient in atrial fibrillation. sPLA2-V was absent in the patient in sinus rhythm. However, in the section corresponding to the atrial fibrillation patient, sPLA2-V was detected (D) around the fibrin thrombus and (E) along the endothelial lining. This research was originally published in Blood. López-Sagaseta et al. sPLA2-V inhibits EPCR anticoagulant and antiapoptotic properties by accommodating lysophosphatidylcholine or PAF in the hydrophobic groove. Blood 2012; 119: 2914-21. © the American Society of Hematology.

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