Apolipoprotein A-I enhances activated protein C cytoprotective activity

Eimear M. Gleeson,1,* Aisling M. Rehill,2,* Orla Willis Fox,2 Fionnuala Ni Ainle,3 Cormac J. McDonnell,2 Hannah J. Rushe,2 Seána McCluskey,2 James S. O’Donnell,1,2,4 and Roger J. S. Preston1,2

1National Children’s Research Centre, Our Lady’s Children’s Hospital Crumlin, Crumlin, Dublin, Ireland; 2Irish Centre for Vascular Biology, School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, Dublin, Ireland; 3SPHERE Research Group, School of Medicine, University College Dublin, Dublin, Ireland; and 4St. James’s Hospital, Dublin, Ireland.

Key Points

- High-density lipoprotein and apolipoprotein A-I enhance activated protein C cytoprotective activity.
- High-density lipoprotein and apolipoprotein A-I significantly increase the rate at which activated protein C degrades cytotoxic extracellular histones.

Introduction

Activated protein C (APC) is a crucial regulator of blood clot formation, which it achieves by proteolytic degradation of procoagulant-activated cofactors factor V and factor VIII.1 Separately, APC can initiate cell signaling in numerous cell types to enhance cytoprotective responses to stress or toxic agents.2-4 APC cytoprotective activity on endothelial cells is mediated by agonism of protease-activated receptors (PARs), in particular PAR1 and PAR3, which it activates via proteolysis of defined extracellular cleavage sites.1,5,6 APC cannot efficiently cleave PARs unless already bound to other membrane receptors such as endothelial cell protein C receptor (EPCR),6 Mac-1,7 or apolipoprotein E receptor 2.8 Moreover, APC anti-inflammatory activity is at least in part related to its ability to neutralize cytotoxic extracellular histones released by dying tissue and neutrophil extracellular traps.9 Nevertheless, the molecular basis of how APC facilitates histone proteolysis to limit vascular injury is not well understood.

Despite significant evidence implicating the importance of PAR signaling and extracellular histone proteolysis in mediating the therapeutic benefits of APC,10-12 several aspects of how APC confers protection remain unresolved. In particular, APC is a relatively poor PAR1 agonist and is unlikely to be generated in vivo at the APC concentrations required to induce PAR1 signaling or extracellular histone proteolysis in vitro.9,13 Consequently, we sought to investigate whether endogenous blood-borne factors may play a role in regulating PAR1 signaling and extracellular histone proteolysis by APC.

Methods

Materials

Recombinant human APC was generated and characterized as previously described.13 Human thrombin was purchased from Haematologic Technologies Inc. Human plasma–purified high-density lipoprotein (HDL; >95% pure, #LP3-5MG), apolipoprotein A-I (Apo A-I; >95% pure, #ALP10-M), and Apo A-II (>95% pure, #A0792) were purchased from Merck-Sigma. HDL was isolated by sequential flotation ultracentrifugation and was composed of 55% to 45% lipid and 45% to 55% protein. HDL was refrigerated and used fresh, as loss of activity was observed when frozen or after prolonged storage, in keeping with previous reports.14 Escherichia coli–expressed recombinant Apo A-I (>95% pure, #SRP4693) was purchased from Merck-Sigma. Recombinant histone H3.1 was purchased from New England Biolabs, and antihistone H3.1 antibody (5192S) was purchased from Cell Signaling Technology. Phospholipid MP-Reagent (TS60.00) was purchased from Thrombinscope. Anti-EPCR antibody RCR-252 and PAR1 antagonist (SCH530348) were purchased from Sigma and R&D Systems, respectively. The S1P1 receptor antagonist (Ex 26) was purchased from Bio-Techne.

Assessment of endothelial cell barrier permeability

Endothelial cell barrier permeability was determined as previously described.13,15 Briefly, EA.hy926 cells (ATCC) or human umbilical vein endothelial cells (PromoCell) were grown to confluence on polycarbonate membrane transwells (Merck) and incubated with APC and other reagents as indicated.
Antibodies or antagonists when used were incubated with cell monolayers for 30 minutes before addition of APC. After 3 hours, cells were treated with thrombin (5 nM) for 10 minutes, after which the cells were washed and incubated with 0.67 mg/mL Evans Blue with 4% bovine serum albumin (Sigma). Changes in endothelial cell barrier permeability were then determined as previously described.15

Visualization of histone proteolysis by APC

Recombinant histone H3.1 (New England Biolabs) was incubated with APC in the presence or absence of Apo A-I or HDL at stated concentrations for 2 hours at 37°C. Histone proteolysis was assessed by immunoblotting using an antihistone H3.1 antibody (5192S) that detects remaining intact or cleaved histone H3.1.

Results and discussion

HDL is an abundant plasma lipoprotein that has previously been reported to enhance APC anticoagulant activity via acceleration of APC-mediated factor Va proteolysis.16 To determine whether HDL can also modulate non-anticoagulant APC functions such as protection of endothelial barrier integrity in response to thrombin-induced hyperpermeability, endothelial cells were exposed to HDL, and its ability to mediate APC-barrier protective function was assessed. Interestingly, HDL, but not low-density lipoprotein (LDL), markedly enhanced APC barrier-protective function, such that half-maximal barrier protection occurred at five- to 10-fold lower APC concentrations compared with when HDL was absent (Figure 1A-B). Importantly, HDL alone exhibited negligible ability to limit endothelial barrier permeability in the absence of APC. HDL titration revealed that half-maximal enhancement of APC barrier function was achieved at 0.5 mg/mL HDL (Figure 1C), which is within the normal concentration range of plasma HDL (0.4-0.6 mg/mL). These data highlight a potential role for HDL in the protection of inflamed vasculature via enhancement of APC signaling.

We next sought to investigate the molecular basis for HDL enhancement of APC cytoprotective activity, and therefore tested
whether Apo A-I and Apo A-II, 2 abundant protein components found within HDL, might also mediate a similar effect to HDL when tested in purified form. Apo A-I but not Apo A-II was found to replicate the enhanced APC-dependent barrier function observed when HDL was coinubicated with HDL (Figure 1D). In addition, Apo A-I did not affect APC auto-degradation (supplemental Figure 1). To ensure that Apo A-I also mediated similar enhancement on primary endothelial cells, the same experiment was performed on human umbilical vein endothelial cells (Figure 1E). Similarly, no protection of endothelial barrier integrity from thrombin was observed in the presence of Apo A-I alone; however, Apo A-I significantly enhanced APC-mediated barrier protection, as before. Half-maximal barrier protection against thrombin-induced permeability was achieved by 50 to 100 μg/mL of Apo A-I, which is well within the normal physiological range for plasma Apo A-I (~1.3 mg/mL) (Figure 1F). Copurified barrier-protective lipids were not responsible for the observed enhanced endothelial barrier protection.17,18 as both plasma-purified and recombinant Apo A-I exhibited identical enhancement of APC barrier protective function (Figure 1G).

Multiple studies have described the requirement for APC–EPCR binding to enable PAR1 signaling and protection of the endothelium from thrombin-induced barrier leakage.17,19,20 To assess how Apo A-I affects these requirements, we first performed the same assays in the presence of a PAR1 antagonist that blocks PAR1 signaling by APC (Figure 2A). The PAR1 antagonist blocked APC-mediated barrier protection irrespective of the presence of either HDL or Apo A-I. Similarly, an APC mutant with defective ability to mediate PAR1 proteolysis (APC330E)20 remained ineffective in the presence of Apo A-I or HDL (Figure 2B). These data suggest that HDL or Apo A-I enhancement of APC cytoprotective activity on endothelial cells remains dependent on functional PAR1 signaling.

To investigate the requirement for APC–EPCR binding in Apo A-I–enhanced APC barrier protective function, barrier integrity was
assessed in the presence of an anti-EPCR antibody (RCR-252) that blocks APC–EPCR binding and subsequent EPCR-dependent PAR1 proteolysis and signaling. As expected, the anti-EPCR antibody completely ablated the APC barrier-protective function. However, in the presence of Apo A-I or HDL, the requirement for EPCR binding was obliterated, and APC barrier-protective function proceeded at an enhanced rate compared with wild-type APC (Figure 2A). To ensure that continued APC cytoprotective signaling did not occur due to residual APC–EPCR binding even in the presence of RCR-252, we compared the Apo A-I–enhanced barrier-protective function of wild-type APC with an APC mutant with defective EPCR-binding affinity (APC^{LBF}). APC^{LBF} was unable to mediate barrier protection from thrombin-induced barrier permeability. However, in the presence of either HDL or Apo A-I, APC^{LBF} regained the ability to mediate protection against thrombin-induced barrier leakage and exhibited a similarly enhanced protective activity as wild-type APC (Figure 2C). Moreover, because sphingosine 1-phosphate receptor 1 (S1PR1) is essential for APC protection of endothelial barrier integrity,^{17,18} we evaluated APC protection from thrombin-induced barrier leakage in the presence of an S1PR1 inhibitor (Ex 26) but found that this impaired endothelial barrier protection by APC, both in the presence and absence of Apo A-I (supplemental Figure 2). These data indicate that the presence of Apo A-I promotes APC endothelial cell barrier protection from disruption using a mechanism that requires functional PAR1 and S1PR1 but not EPCR. This finding suggests that EPCR may not always be required for APC-induced endothelial cell barrier protection from thrombin-induced damage when Apo A-I/HDL is present.

Having shown that HDL and Apo A-I could increase PAR1-dependent endothelial barrier-protective activity, we next assessed whether HDL and Apo A-I could also modulate degradation of extracellular histones by APC. HDL was found to dose dependently enhance histone proteolysis by APC (Figure 2D). Incubation with Apo A-I alone had no effect (Figure 2E); however, coincubation of APC with Apo A-I caused significantly enhanced proteolysis of histone H3.1 by APC. Anionic phospholipid vesicles (PL) have previously been shown to increase histone proteolysis by APC^{19} but reportedly do not contribute to HDL enhancement of APC anticoagulant activity.^{14} Enhanced histone proteolysis by APC in the presence of Apo A-I was impaired by the presence of PL (Figure 2F), indicating Apo A-I and PL may compete for shared binding sites on APC.

Collectively, these studies describe a novel role for the Apo A-I component of HDL in enhancing APC inhibition of vascular dysfunction caused by endogenous barrier-disruptive and cytotoxic agents. The ability of Apo A-I/HDL to enhance multiple aspects of APC activities, including its anticoagulant, endothelial cell signaling and histone proteolytic activity, indicates that enhancement may be mediated by modulation of its proteolytic activity and suggests this activity may contribute to the beneficial therapeutic outcomes associated with APC administration.^{10–12} Moreover, the ability of HDL and Apo A-I to modulate APC endothelial cytoproteective activity suggests a potential role in modulating cardiovascular health outcomes in people with low HDL levels.

**Acknowledgments**

This study was supported by a Science Foundation Ireland Career Development Award (15/CDA/3499), a Technology and Innovation Development Award (18/TIDA/6001), and a National Children’s Research Centre Project Award (C/18/3).

**Authorship**

Contribution: All authors contributed to the experimental design, performed experiments and data analysis, and assisted with manuscript preparation and revision.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID iDs: F.N.A., 0000-0003-0163-792X; S.M., 0000-0001-9245-1447; J.S.O., 0000-0003-0309-3313; R.J.S.P., 0000-0003-0108-4077.

Correspondence: Roger J. S. Preston, Irish Centre for Vascular Biology, School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, 123 St Stephen’s Green, Dublin 2, Ireland; e-mail: rogerpreston@rcsi.ie.

---

**References**

1. Griffin JH, Fernández JA, Gale AJ, Mosnier LO. Activated protein C. J Thromb Haemost. 2007;5(suppl 1):73-80.
2. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C: biased for translation. Blood. 2015;125(19):2898-2907.
3. Shahzad K, Kohli S, Al-Dabet MM, Isermann B. Cell biology of activated protein C. Curr Opin Hematol. 2019;26(1):41-50.
4. McDonnell CJ, Soule EE, Walsh PT, O’Donnell JS, Preston RJ. The immunoregulatory activities of activated protein C in inflammatory disease. Semin Thromb Hemost. 2018;44(2):167-175.
5. Mosnier LO, Sinha RK, Burnier L, Bouwens EA, Griffin JH. Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. Blood. 2012;120(26):5237-5246.
6. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science. 2002;296(5574):1880-1882.
7. Cao C, Gao Y, Li Y, Antalis TM, Castellino FJ, Zhang L. The efficacy of activated protein C in murine endotoxemia is dependent on integrin CD11b. J Clin Invest. 2010;120(6):1971-1980.
8. Sinha RK, Yang XV, Fernández JA, Xu X, Mosnier LO, Griffin JH. Apolipoprotein E receptor 2 mediates activated protein C-induced endothelial Akt activation and endothelial barrier stabilization. Arterioscler Thromb Vasc Biol. 2016;36(3):518-524.
9. Xu J, Zhang X, Pelayo R, et al. Extracellular histones are major mediators of death in sepsis. Nat Med. 2009;15(11):1318-1321.
10. Kerschen E, Hernandez I, Zogg M, et al. Activated protein C targets CD8+ dendritic cells to reduce the mortality of endotoxemia in mice. *J Clin Invest.* 2010;120(9):3167-3178.

11. Sinha RK, Wang Y, Zhao Z, et al. PAR1 biased signaling is required for activated protein C in vivo benefits in sepsis and stroke. *Blood.* 2018;131(11):1163-1171.

12. Bock F, Shahzad K, Wang H, et al. Activated protein C ameliorates diabetic nephropathy by epigenetically inhibiting the redox enzyme p66Shc. *Proc Natl Acad Sci U S A.* 2013;110(2):648-653.

13. Gleeson EM, McDonnell CJ, Soule EE, et al. A novel protein C-factor VII chimera provides new insights into the structural requirements for cytoprotective protease-activated receptor 1 signaling. *J Thromb Haemost.* 2017;15(11):2198-2207.

14. Fernandez JA, Deguchi H, Banka CL, Witztum JL, Griffin JH. Re-evaluation of the anticoagulant properties of high-density lipoprotein-brief report. *Arterioscler Thromb Vasc Biol.* 2015;35(3):570-572.

15. Gleeson EM, Dichiara MG, Salicio A, et al. Activated protein C β-glycoform promotes enhanced noncanonical PAR1 proteolysis and superior resistance to ischemic injury. *Blood.* 2015;126(7):915-919.

16. Griffin JH, Kojima K, Banka CL, Curtiss LK, Fernández JA. High-density lipoprotein enhancement of anticoagulant activities of plasma protein S and activated protein C. *J Clin Invest.* 1999;103(2):219-227.

17. Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood.* 2005;105(8):3178-3184.

18. Finigan JH, Dudek SM, Singleton PA, et al. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. *J Biol Chem.* 2005;280(17):17286-17293.

19. Bae JS, Yang L, Manithody C, Rezaie AR. The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood.* 2007;110(12):3909-3916.

20. Yang L, Bae JS, Manithody C, Rezaie AR. Identification of a specific exosite on activated protein C for interaction with protease-activated receptor 1. *J Biol Chem.* 2007;282(35):25493-25500.