To the editor:

Factor X binding to endothelial cell protein C receptor: comparison with factor VIIa and activated protein C

Endothelial cell protein C receptor (EPCR) is the cellular receptor for protein C (PC) and activated protein C (APC). Studies from our laboratory and others have shown that FVII/FVIIa also binds to EPCR in a true-ligand fashion. Recently, it has been suggested that FX and FXa can bind to EPCR and these interactions may play a role in regulating TF-FVIIa-FXa or FXa-mediated cell signaling via activation of protease activated receptors (PARs). This reported observation that FX/FXa binds to EPCR is somewhat surprising given that the specific Gla domain residues of protein C necessary for molecular recognition of EPCR are not completely conserved in human FX. As reported recently, if FXa binds to EPCR with an affinity similar to that of APC binding to EPCR, then this interaction may not only influence the activation of FX and FXa-mediated cell signaling, but could also modulate EPCR-dependent functions of APC and FVIIa by competing with APC and FVIIa for EPCR binding.

To evaluate the physiologic relevance of FX binding to EPCR, we have reexamined FX binding to EPCR on endothelial as well as CHO-EPCR cells using plasma concentrations of FX and multiple experimental approaches. Radioligand binding studies did not indicate any significant difference in the binding of FX or FXa between CHO and CHO-EPCR cells (Figure 1A). Furthermore, pretreatment of CHO or CHO-EPCR cells with an EPCR blocking mAb did not diminish the basal binding of FX or FXa to these cells.

Figure 1. Binding of factors X, Xa, VIIa, and APC to EPCR expressing cells. (A) CHO cells (black bars) or CHO cells stably transfected to express human EPCR (CHO-EPCR; gray bars) were incubated with 125I-labeled human FVIIa (10nM and 50nM), human APC (50nM), human FXa (150nM), or human FX (150nM) in HEPES buffer (10mM HEPES, 0.15M NaCl, 4mM KCl, and 11mM glucose) containing CaCl2 (5mM), MgCl2 (1mM), and BSA (1 mg/mL) for 3 hours at 4°C. At the end of incubation, cells were washed 4 times with the same buffer and surface bound ligands were eluted with glycine (100mM, pH 2.3) and counted for radioactivity to determine the amount of ligand bound to the cells. (B) Same as panel A except that CHO and CHO-EPCR cells were replaced with HUVECs pretreated with EPCR blocking mAb (black bars) or control vehicle (gray bars), respectively. (C) CHO or CHO-EPCR cells were incubated with various concentrations of biotinylated active site-blocked FVIIa, APC (ΔA) or FXa (ΔA) in Ca2+/Mg2+ containing buffer for 3 hours at 4°C. After washing the cells to remove unbound ligands, the surface bound ligands were detected by fixing the cells and adding alkaline phosphatase coupled streptavidin followed by BluePhos phosphatase substrate system (KPL). EPCR-specific binding was calculated by subtracting the absorbance measured in CHO cells from that of CHO-EPCR cells. Biotinylated active site-inhibited FXa, FVIIa, or APC were prepared by incubating FXa, FVIIa, or APC with 10-fold molar excess of biotinylated EGR for 3 hours at room temperature and excess probe was removed by dialysis. (D) Same as panel C except that CHO and CHO-EPCR cells were substituted with HUVECs pretreated with EPCR blocking mAb or a control vehicle, respectively, and EPCR-specific binding was calculated by subtracting the absorbance obtained with HUVECs pretreated with EPCR blocking antibody from HUVECs not treated with the antibody. (E) CHO-EPCR cells were incubated with 125I-FVIIa (10nM) in the presence of various concentrations of unlabeled competitors, FIX (ΔA), prothrombin (PT, ΔA), FX (ΔA), FVIII (ΔA), APC (ΔA) for 3 hours at 4°C. The surface bound 125I-FVIIa was eluted with low pH glycine and counted for radioactivity. (F) HUVECs, treated EPCR blocking mAb (25 μg/mL) or control vehicle (top panel) or CHO-EPCR cells (bottom panel) were exposed to FVIIa (10 nM FVIIa, 50 nM APC, 70 nM FX, 175 nM) and blood samples were drawn retroorbitally pre- and postadministration of APCi. Mouse protein C level in plasma was measured using mouse protein C–specific ELISA (G) and mouse FX level was measured in a FX specific clotting assay (H).
(data not shown). Similarly, blockade of EPCR on HUVECs with this EPCR blocking mAb did not have a significant effect on FX or FXa binding (Figure 1B). FVIIa and APC binding studies performed in parallel with FX or FXa experiments clearly demonstrated that both FVIIa and APC bound to cells to a similar degree and in an EPCR-specific manner (Figure 1A-B). Analysis of the binding of biotinylated, active-site blocked FXa, FVIIa and APC to EPCR on CHO-EPCR and HUVECs revealed that little FXa was bound to EPCR on cell surfaces compared with FVIIa or APC (Figure 1C-D). In this assay, FVIIa and APC both bound to EPCR with an apparent Kd of ~15 to 25nM, whereas the Kd for FXa was ~1μM. Consistent with these data that FX does not bind appreciably to EPCR, even a 100-fold molar excess of unlabeled FX (1μM) failed to compete effectively with the binding of 125I-FVIIa (10nM) to CHO-EPCR cells (Figure 1E). Analysis of FX binding to EPCR expressing cells by confocal fluorescence microscopy did not show any detectable fluorescence, either at the cell surface or intracellularly, in CHO-EPCR cells or HUVECs exposed to FX tagged with a fluorescence dye (AF488; Figure 1F). In additional studies, we measured plasma levels of mouse factor X and protein C in EPCR overexpressing mice that received a high dose of active-site inhibited human APC. EPCR overexpression has been found to decrease circulating levels of protein C, while administration of human protein C has been shown to decrease circulating levels of protein C, while administration of human protein C has been shown to increase mouse protein C levels in circulation by displacing endogenous protein C from EPCR on the endothelium. As expected from this study, administration of human APC increased plasma levels of mouse protein C (Figure 1G). In contrast to protein C, there was not a detectable increase in plasma levels of mouse FX after APC administration (Figure 1H). These data indicate that FX does not effectively interact with EPCR in vivo, at least in regards to the mouse system. Overall our data indicate that FX binding to EPCR, if any, is minimal and likely physiologically insignificant. Our findings do not exclude the possibility that FX/FXa could indirectly interact with EPCR as suggested by others.

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To the editor:

Linking air pollution exposure with thrombosis

We read with interest the review by Franchini and Mannucci examining the link between exposure to particulate matter air pollution (PM) and an increased tendency toward thrombosis. In their discussion of the potential mechanisms by which PM might induce thrombosis, the investigators highlight the excellent work by Nemmar and colleagues suggesting that PM enhances the release of histamine by mast cells and the resulting activation of platelets increases the tendency toward thrombosis in hamsters administered intratracheal suspensions of diesel exhaust particles. We were surprised that the authors did not discuss an additional mechanism. In mice, we reported that the intratracheal administration of fine urban particulates or the inhalation of concentrated ambient particulate matter air pollution from Chicago resulted in an increase in the plasma levels of thrombin-antithrombin (TAT) complexes and accelerated arterial thrombosis in the ferric chloride carotid injury model via a mechanism that required the release of IL-6 from alveolar macrophages. This mechanism is attractive as resident macrophages in the lung are likely the “first responders” to inhaled particles and the prothrombotic effects of IL-6 have been
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