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Role of the protein C pathway in the extra-intestinal thrombosis associated with murine colitis

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Abstract

Background & Aims—Chronic inflammatory bowel diseases (IBD) are associated with an increased risk for thromboembolism. While thrombosis is known to contribute to the morbidity and mortality of patients with IBD, the underlying mechanisms that contribute to the genesis of a hypercoagulable state during intestinal inflammation remain poorly defined. The objective of this study was to determine whether the protein C pathway contributes to the enhanced extra-intestinal thrombosis that is associated with dextran sodium sulfate (DSS)-induced colitis in mice.

Methods—Microvascular thrombosis was induced in cremaster muscle microvessels of normal and colitic mice using a light/dye injury model. DSS colitis enhanced thrombus formation in cremaster arterioles of wild type (WT) mice.

Results—The DSS-induced thrombosis response was greatly attenuated in transgenic mice overexpressing the endothelial protein C receptor (EPCR-TgN). Activated protein C (APC), administered to colitic WT mice immediately prior to photoactivation, also afforded protection against thrombosis, while an anti-APC antibody enhanced thrombus formation.

Conclusions—These findings indicate that elevated APC levels, derived from either endogenous or exogenous sources, confer protection against the extra-intestinal thrombosis that accompanies colonic inflammation.

Keywords

inflammatory bowel disease; activated protein C; arterioles; venules

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Introduction

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are chronic diseases that are associated with intense intestinal inflammation, gut mucosal injury, and thrombosis [1, 2], with the latter response occurring both within the bowel wall and distant sites [3, 4]. There is a growing body of evidence that the inflammatory and thrombotic responses are linked, with inflammation promoting thrombosis and vice versa[5]. Support for a link between two these processes is provided by recent work implicating the downregulation of the anticoagulant system of endothelial protein C receptor (EPCR) and thrombomodulin (TM) in human and experimental IBD [6]. It was shown that EPCR and TM expression are reduced in IBD, suggesting decreased activation of protein C. Treatment of cytokine-activated human intestinal microvascular endothelial cells (HIMEC) with recombinant activated protein C (rAPC) resulted in reductions in endothelial cell chemokine production and expression of adhesion molecules [6]. Furthermore, disease severity in mice with dextran sodium sulfate (DSS) induced colitis was significantly reduced following treatment with rAPC, as reflected by reductions in body weight loss, disease activity index, and histopathological changes [6].

The significant contribution of the protein C pathway to the inflammatory and tissue injury responses of the gut in experimental IBD raises the possibility that downregulation of this anticoagulant mechanism may also contribute to the prothrombogenic state that is associated with IBD. The protein C anticoagulant pathway, which is initiated when thrombin binds to TM on endothelial cells, is a major line of defense against microvascular thrombosis [7]. Thrombin-TM complexes activate protein C via a proteolytic process that is accelerated over 10-fold by engagement of the endothelial protein C receptor [8]. Once APC dissociates from the EPCR it can interact with protein S to inactivate factors Va and VIIIa[7]. Since the expression of EPCR and TM are significantly downregulated by cytokines (TNF α, IL-1 β) that exhibit increased expression and plasma levels in IBD [9, 10], it is possible that inactivation of the protein C pathway also accounts for the enhanced thrombogenesis that is associated with IBD.

The overall objective of this study was to determine whether genetic, pharmacologic, or immunologic manipulation of the protein C pathway alters the enhanced extra-intestinal thrombogenesis that is associated with experimental IBD. To achieve this objective, the light/dye method was used to induce microvascular thrombosis in arterioles and venules of the cremaster muscle in mice with DSS-induced colitis. The thrombosis responses in microvessels of wild type (WT) colitic mice were compared to those observed in mutant mice that overexpress the endothelial protein C receptor (EPCR-TgN), and in WT mice receiving either exogenous murine APC or a monoclonal antibody that blocks APC. Our findings support a role for the protein C pathway in the extra-intestinal thrombosis associated with experimental IBD.

Materials & Methods

Mice

Male C57BL/6 (wild-type [WT] control strain) mice (Jackson Laboratories, ME) or transgenic mice overexpressing the endothelial protein C receptor (EPCR-TgN) (Oklahoma Medical Research Foundation, OK) were used. The EPCR-TgN transgenic mice were backcrossed into C57BL/6 mice and exhibit normal size, weight, viability, fertility, blood cell count and chemistries [11]. EPCR protein levels in all organs of the transgenic mice are at least eight-fold higher than in their WT counterparts. There was no evidence of thrombotic or hemorrhagic complications, or microscopic tissue abnormalities in EPCR-TgN mice [11]. A total of 57 WT (weight: 24.5 ± 0.4 g) and 18 EPCR transgenic (weight: 24.2 ± 0.6 g) mice were employed in this study. All mice were housed under specific pathogen-free (SPF) conditions in standard cages and fed standard laboratory chow and water until the desired age (6–8 week). All

experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee of LSU Health Sciences Center and performed according to the criteria outlined by the National Institutes of Health.

DSS-induced colitis

Colitis was induced, as previously described [12], by feeding mice 3 % (wt/vol) dextran sodium sulfate (mol wt, 40,000; ICN Biomedicals) dissolved in filter-purified drinking water. The first day of DSS feeding was defined as day-0 and the mice were maintained on the DSS until day-6. The 3 % DSS regimen, unlike higher DSS doses, is not associated with mortality. Control mice received filtered water alone (without DSS).

Assessment of inflammation in DSS-treated mice

Body weights, fecal status, presence of occult blood in the stools and peri-anal bleeding were evaluated and recorded every day while the mice received DSS. Occult blood was detected using guaiac paper (ColoScreen®; Helena Laboratories) [12]. A disease activity index (DAI), a measure of disease severity ranging between 0 and 4, was calculated from data collected on stool consistency, presence or absence of fecal blood, and weight loss, as previously described [12]. The DAI was monitored to ensure that DSS treatment resulted in clinical responses that are consistent with disease activity.

Cremaster muscle preparation

On day-6 of DSS (colitis) or water (control) treatment, mice were anesthetized using 50 mg/kg body wt (i.p.) pentobarbital, with supplemental doses of 12.5 mg/kg, given as needed. The right internal jugular vein was cannulated for intravenous administration of FITC dextran. Body temperature was maintained at 35.5–36.5 °C during the entire experiment with a homeothermic blanket and monitored with a rectal temperature probe. The cremaster muscle was prepared for intravital fluorescence microscopic observation, as previously described[13]. The surface of the exposed cremaster muscle was suffused continuously with bicarbonate-buffered saline (BBS), with a pH 7.35–7.45. The cremaster preparation was moved onto the stage of an upright fluorescent microscope and allowed to stabilize for 20–30 min before initiation of the experiment. Microscopic observations were carried out using an upright microscope (BX51WI, Olympus, Japan) with a 40X water immersion objective lens (LUMPlanFI/IR 40x 0.80 w, Japan). The microscopic image was projected onto a monitor (Sony TRINITRON PVM-2030, Japan) through a color video camera (Hitachi VK-C150, Japan) and recorded using a DVD recorder (JVC SR-MV50, NJ). A video timer (Panasonic Time-Date Generator WJ-810, Japan) was connected to the monitor to record time and date. The diameters of the cremaster vessels were measured by video analysis software (ImageJ 1.37v, NIH, Public Domain software) on a personal computer (G4 Macintosh, Apple, CA).

After the preparation was stabilized, 10 mL/kg of 5 % FITC-dextran (150,000 MW, Sigma, MO) was slowly injected intravenously. It was allowed to circulate for 10 min, and then second- or third-order unbranched venules and arterioles [14] with diameters (D_V) ranging between 30–50 μm were selected for study. Red blood cell velocity (V_{RBC}) in the microvessels was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A & M University, College Station, TX). Blood flow was calculated from the product of mean red blood cell velocity [$V_{mean} = V_{RBC}/1.6$] and cross-sectional area, assuming cylindrical geometry. Wall shear rate (WSR) was calculated on the basis of the Newtonian definition: $WSR = 8 (V_{mean} / D_V)$ [15]. The fluorescent microscopic images were received by a charge-coupled device (CCD) video camera (Hamamatsu XC-77, Japan).

Light/dye-induced thrombosis

Photoactivation of FITC-dextran (excitation: 495 nm, emission: 519 nm) within the microvessels was performed after the fluorochrome was in the circulation for 10 minutes. Less than 100 μm of vessel length was exposed to epi-illumination using a 175-W xenon lamp (Lambda LS, Sutter, CA) and a fluorescein filter cube (HQ-FITC, Chroma, VT). The excitation power density was measured daily (ILT 1700 Radiometer, SED033 detector, International Light, MA) and maintained within 1 % of 0.77 W/cm^2, as previously described [16, 17, 18]. Epi-illumination was continuously applied to the vessels and thrombus formation was quantified by determining: 1) the time of onset of platelet deposition/aggregation within the microvessel (onset time), and 2) the time required for complete flow cessation for ≥ 60 sec (cessation time). Epi-illumination was discontinued once blood flow ceased in the vessel under study. Typically, 2 – 4 thrombi were induced in each mouse and the results of each vessel type (venules, arterioles) were averaged.

Experimental protocols

In order to determine whether DSS-induced colitis enhances thrombosis in a distant vascular bed (cremaster) and whether the protein C anticoagulant system can modulate this response, the light/dye injury model was used to monitor thrombus formation in the following experimental groups: 1) control wild type (WT) mice (no DSS in drinking water), 2) DSS-treated WT mice, 3) water-treated EPCR-TgN mice, 4) DSS-treated EPCR-TgN mice, 5) water-treated WT mice receiving 10 μg murine activated protein C (APC) 10 min before vessel epi-illumination[19], 6) DSS-treated WT receiving 10 μg APC 10 min before vessel epi-illumination, 7) water-treated WT mice receiving 10 mg/kg of rat anti-mouse APC monoclonal antibody (APC mAb 1609) 20 min prior to vessel epi-illumination[20], and 8) DSS-treated WT mice receiving 10 mg/kg of the APC mAb.

Statistics

Data were analyzed using standard statistical analysis, i.e., one-way ANOVA and Fisher's post hoc test. All values are reported as means ± SE from 8–12 mice, and statistical significance was set at $P < 0.05$.

Results

Exposure of the cremaster to epi-illumination for 30 min in the absence of FITC-dextran did not induce any signs of platelet aggregation and thrombus formation in either venules or arterioles; nor did it alter microvessel red blood cell velocities or shear rate. Table 1 summarizes the values for wall shear rate that were determined in arterioles and venules of all experimental groups immediately prior to the induction of light/dye injury. No significant differences in wall shear rate were noted between groups for either arterioles or venules.

Figure 1 illustrates the changes in the time of onset of thrombosis and time to flow cessation in cremaster venules and arterioles of control and colitic (DSS-treated) WT mice. The time required to elicit both the onset of thrombosis (5.92 ± 1.16 vs 0.55 ± 0.15 min) and complete flow cessation (29.61 ± 1.97 vs 3.50 ± 0.58 min) with light/dye injury was markedly longer in arterioles than in venules, as previously reported by others [17,18]. While no differences in onset and flow cessation times were noted in venules of control and colitic mice, the arterioles of colitic mice exhibited large and significant reductions in both variables (onset and flow cessation times) compared to their control counterparts, suggesting that experimental colitis enhances thrombus formation in arterioles but not in venules. The selective effects of DSS colitis on thrombus formation in arterioles is consistent with previously reported results from our laboratory.[18]

Figure 2 compares the thrombosis responses to light/dye injury in arterioles and venules of untreated and DSS-treated groups of wild type mice and endothelial protein C receptor overexpressing transgenic mice. In both arterioles and venules, the onset of thrombus formation did not differ between colitic WT mice and colitic EPCR-TgN mice or between control WT mice and untreated EPCR-TgN mice. However, the time to flow cessation was significantly increased in the microvessels (arterioles & venules) of colitic EPCR-TgN compared to WT mice with DSS-induced colitis. The time to flow cessation did not different between venules of control EPCR-TgN and colitic EPCR-TgN mice. Venules exhibited more pronounced increases in the time required for complete flow cessation than arterioles in the untreated and DSS-treated EPCR-TgN. These findings indicate that over-expression of endothelial protein C receptors protects both arterioles and venules of colitic mice from thrombus-induced flow cessation.

Since EPCR over-expression exerted a marked influence on time to flow cessation following light/dye injury in cremaster microvessels of colitic mice, we examined whether similar protection would be afforded to wild type mice treated with APC. Figure 3 compares the thrombosis responses to light/dye injury in cremaster arterioles and venules of untreated and APC-treated WT (control) and colitic mice. No statistically significant differences were noted between control WT and control WT with APC treatment or between colitic WT and colitic WT with APC treatment. While exogenous APC did not delay the time to flow cessation in arterioles of control (non-colitic) WT mice, it significantly prolonged the time required for blood flow cessation in colitic mice. In venules, the APC-induced increment in time to flow cessation was far more dramatic compared to untreated WT mice. Overall, the responses to APC treatment were qualitatively similar to those observed in EPCR-TgN mice.

Figure 4 summarizes the effects of treatment with an APC-blocking mAb on the light/dye induced thrombosis responses of cremaster arterioles and venules in control mice and mice with DSS-induced colitis. In venules, immunoneutralization of endogenous APC had no effect on either the onset of thrombosis or the time to flow cessation in the WT controls and WT colitic mice. On the other hand, APC immunoblockade significantly accelerated light/dye-induced thrombosis in colitic mice, as reflected by the even shorter times of onset and flow cessation observed after antibody administration.

Discussion

Although inflammatory bowel diseases (IBD) are associated with an increased risk for thrombosis [1, 2], the mechanisms that underlie the thrombus formation in extra-intestinal tissues during chronic gut inflammation remain poorly understood. However, there is some evidence indicating that alterations in the protein C pathway, an anticoagulant system that limits the generation of thrombin, may contribute to the coagulation abnormalities that accompany IBD. For example, the results of a recent report indicates that the two major forms of human IBD (ulcerative colitis and Crohn's disease) are associated with a reduced expression of EPCR and thrombomodulin in intestinal microvascular endothelium, which impairs the activation of protein C in the microvasculature of the inflamed bowel [6]. This impairment of protein C activation does not appear to be confined to inflamed gut tissue since mice with DSS-induced colitis exhibit a marked reduction in the capacity of plasma to activate exogenously administered protein C [6]. The overall objective of this study was to evaluate the contribution of the protein C pathway to the enhanced thrombus formation observed in cremaster muscle microvessels during experimental colitis. The results of our study are consistent with an altered protein C pathway in experimental IBD and suggest that an impaired protein C pathway accounts for the enhanced thrombus formation in extra-intestinal tissue during chronic intestinal inflammation.

One experimental strategy that was used to assess the contribution of EPCR to IBD-enhanced thrombosis was to evaluate light/dye-induced thrombus formation in colitic EPCR-TgN mice, in which the expression of EPCR is under the control of the Tie2 promoter [11]. Consequently, these mice express high levels of EPCR on endothelial cells in all vascular beds. They exhibit a normal phenotype and show no signs of spontaneous hemorrhage or thrombosis [11]. EPCR-TgN mice generate more APC in response to thrombin and are resistant to factor Xa-induced thrombosis [11]. When DSS colitis was induced in EPCR-TgN mice, we noted a significant increase in the time required for light/dye-activated arterioles to manifest thrombosis-induced flow cessation. Similar protection against light/dye-induced thrombosis in cremaster arterioles was noted in WT colitic mice receiving murine APC, providing additional support for the protein C pathway in the IBD associated thrombotic response.

A third strategy that was used to assess the involvement of the protein C pathway in the colitis-induced enhancement of light/dye-induced microvessel thrombosis was APC immunoblockade. Administration of an anti-mouse APC mAb might be expected to worsen the thrombotic response if sufficient amounts of the anti-coagulant were still available to reduce the severity of the thrombosis elicited by light/dye injury in cremaster arterioles of colitic mice. Indeed, we noted a significant acceleration of both the onset time and time to flow cessation in colitic mice receiving the APC neutralizing antibody. Hence, our findings suggest that the amount of APC that is generated in the muscle microvasculature during DSS colitis is still sufficient to offer a significant level of protection against thrombus formation in arterioles. Low but significant levels of APC in colitic mice would also explain why genetic (EPCR-TgN) or pharmacologic (administration of murine APC) interventions that elevate APC levels would afford protection against thrombus formation, while APC immunoblockade further enhanced thrombosis responses.

While our results implicate the protein C pathway in the extra-intestinal thrombosis associated with IBD, the mechanisms that contribute to the activation of this pathway in an organ distant to the inflamed intestine remain unclear. Changes in microvascular hemodynamics within cremaster microvessels are an unlikely explanation since no difference in shear rate was noted between any of the experimental groups (Table 1). Furthermore, it was previously reported that wall shear rate does not influence the time to onset of thrombosis induced by light/dye injury in either arterioles or venules of mice treated with bacterial endotoxin [16]. Soluble circulating EPCR (sEPCR) may also contribute to the distant thrombosis elicited by gut inflammation. EPCR is shed from activated endothelium and gains access to plasma, where it retains its affinity for both protein C and APC[21]. sEPCR inhibits protein C activation by competing with the membrane form of the receptor on the vessel wall [22] and it also inhibits APC anticoagulant activity by blocking the interaction of APC with negatively charged membrane surfaces[23]. Since its plasma levels are known to be elevated in chronic inflammatory states[24], it is conceivable that endothelial cell-derived sEPCR from the inflamed gut enters plasma and circulates to the cremaster muscle circulation, where it inhibits protein C activation and promotes thrombosis. This possibility seems unlikely in view of: 1) the need for supraphysiological levels of sEPCR to impair protein C activation[23], and 2) our observation that EPCR-TgN mice are protected against thrombosis during DSS-induced gut inflammation despite a >10-fold increase in sEPCR in the plasma of these mutants [11].

A variety of cytokines have been implicated in pathogenesis of IBD, including TNF α and IL-1 β [25]. The levels of these inflammatory mediators increase significantly within the inflamed bowel wall and in systemic blood, where they are delivered to distant tissues. TNF α and IL-β have been shown to downregulate EPCR expression on microvascular endothelial cells and reduce the capacity of these cells to activate protein C [6]. Hence, it is conceivable that an impaired protein C pathway in extra-intestinal tissues results from the elevated circulating levels of cytokines that accompany IBD. This possibility warrants further study.

In a recent study by Scaldaferri and coworkers [6], it was shown that treatment of DSS colitic mice with recombinant APC results in a blunted disease activity index and less histologic injury. The ability of APC to inhibit the gut inflammatory response to DSS raises the possibility that the altered thrombotic responses in cremaster muscle microvessels noted in our studies employing genetic, pharmacological and immunological approaches to manipulate APC levels may be secondary to a diminished inflammatory/injury response in the colon. This is unlikely for the studies employing murine APC or the APC blocking antibody since these reagents were administered minutes before light/dye-induced thrombus formation in mice with a well established colonic inflammatory response. However, the possibility that EPCR-TgN mice exhibited a diminished pro-thrombotic state in cremaster arterioles because of a blunted colonic inflammatory response mediated by increased plasma APC cannot be discounted in view of the work of Scaldaferri et al[6]. In the latter study[6], DSS-treated mice received 1 mg/Kg APC intravenously, which would yield (at least transiently) a plasma APC concentration of 20 ug/ml, assuming a plasma volume of 5%. It has been shown that APC levels are elevated to approximately 1 μg/ml in plasma of EPCR-TgN mice [11] It is possible therefore that the higher APC levels achieved with the intravenous injection protocol may result in a more effective anti-inflammatory action of the anticoagulant.

The results of this study reveal some interesting differences in the responses of arterioles and venules to protein C pathway directed interventions. For example, the venules exhibited more robust changes (when compared to arterioles) in the flow cessation responses in EPCR-TgN mice and in WT mice receiving APC, but did not respond to APC immunoblockade. Little is known about how arterioles and venules differ with respect to coagulation and anticoagulation proteins, adhesion receptors for platelets, and other factors that influence thrombus formation in the microcirculation. Our findings indicate that the basal levels of APC and the vessel wall responses to APC may differ between arterioles and venules, and are consistent with APC level in arterioles that are high enough so that the addition of exogenous APC does not affect cessation time relative to venules, while the APC level in venules is low enough to manifest a response to exogenous APC, but too low to be affected by APC immunoneutralization.

In conclusion, the results of this study indicate that APC, derived from either endogenous or exogenous sources, confers protection against the extra-intestinal thrombosis that accompanies colonic inflammation. Our findings are consistent with a role for the protein C pathway in the extra-intestinal thrombosis associated with colonic inflammation and suggest that APC-directed therapeutic strategies may prove beneficial in reducing the morbidity and mortality from thromboembolic events in IBD patients. Additonal work is needed to more clearly define the mechanism(s) that underlie the dysregulation of APC that is induced by colonic inflammation.

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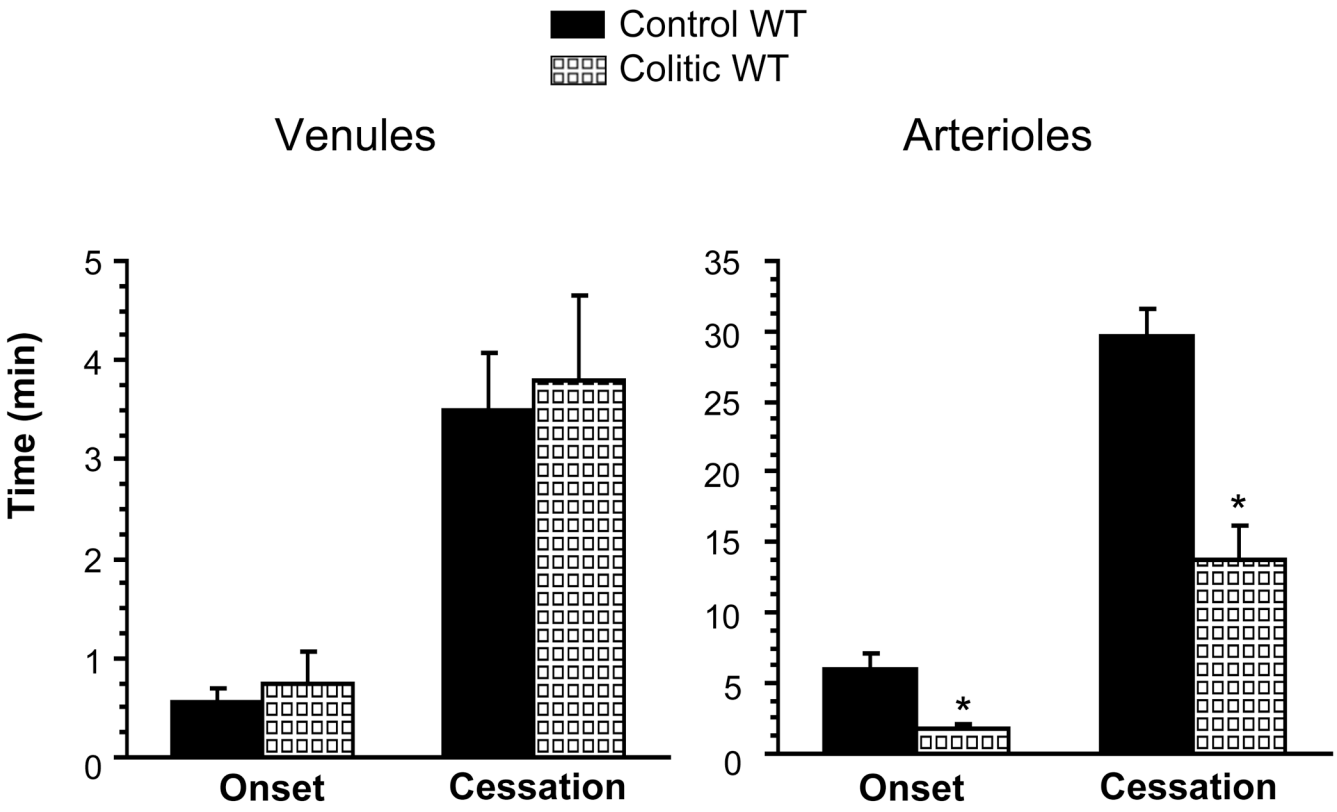

Figure 1.
Time of onset of thrombosis (onset) and time to cessation of flow (cessation) in cremaster venules and arterioles of control (n=10) and colitic (n= 9) wild type mice. Means ± SE. * $p <$ 0.05 vs. control WT.

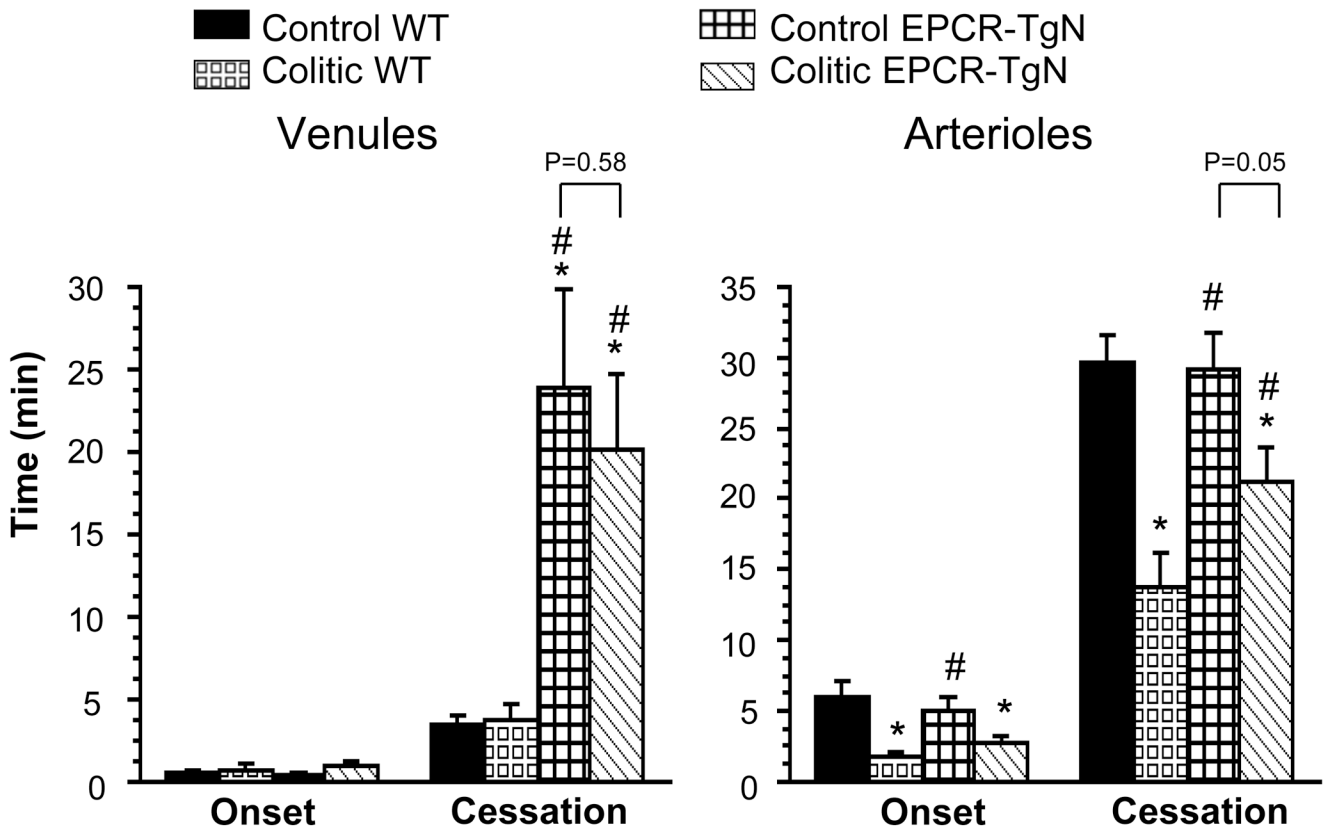

Figure 2.
Effects of endothelial protein C receptor over-expression on thrombus formation in cremaster venules and arterioles of non-colitic and colitic mice. control WT (n = 10), colitic WT (n = 9), non-colitic EPCR-TgN (n= 6) and colitic EPCR-TgN (n = 12). Means ± SE. * $p < 0.05$ vs. control WT. # $p < 0.05$ vs. colitic WT.

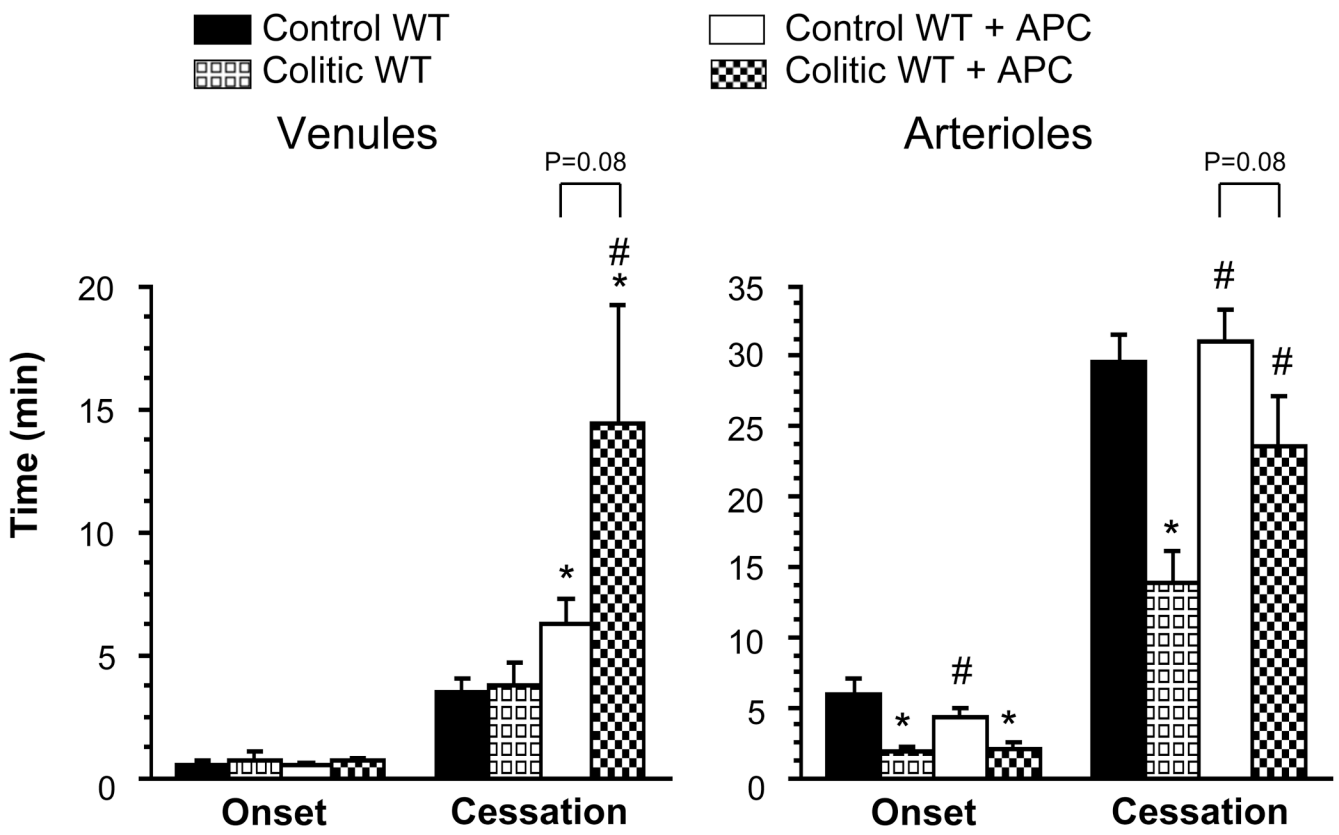

Figure 3.
Effects of APC on light/dye-induced thrombus formation in control (non-colitic) and colitic wild type mice. control (untreated) WT (n = 10), colitic WT (n = 9) control WT + murine APC (n = 12) and colitic WT + APC (n= 11). Means ± SE. * $p < 0.05$ vs. control WT. # $p < 0.05$ vs. colitic WT.

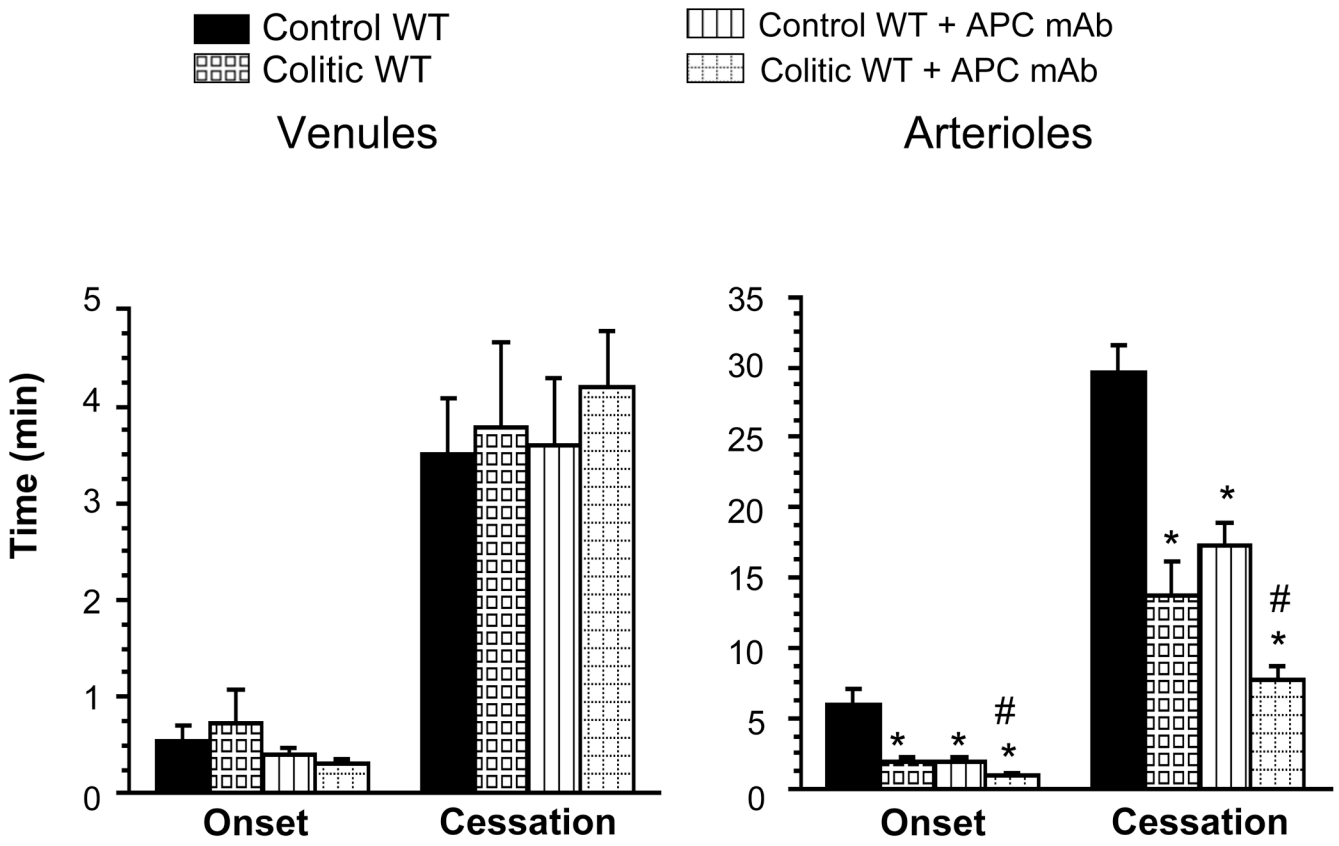

Figure 4.

Effects of activated protein C immunoblockade on the thrombosis responses of venules and arterioles in control (wild type) and wild type mice with DSS-induced colitis. control (non-colitic) WT mice (n = 10), colitic WT (n = 9), control WT + APC mAb (n = 7) and colitic WT + APC mAb (n = 7). Means ± SE. * $p < 0.05$ vs. control WT. # $p < 0.05$ vs. colitic WT.

Table 1

Wall shear rate in venules and arterioles of control (n = 10) and colitic (n = 9) WT mice, non-colitic EPCR-TgN mice (n = 6), colitic EPCR-TgN mice (n = 12), control (n = 12) and colitic (n= 11) WT mice treated with APC, and control (n = 10) and colitic (n = 10) WT mice treated with an APC blocking mAb.

	Wall Shear Rate (s^{-1})	
	Venules	***Arterioles***
Control WT	644.35 ± 27.83	923.80 ± 54.94
Colitic WT *	633.23 ± 49.29	921.77 ± 42.56
Non-colitic EPCR-TgN	645.53 ± 26.58	1157.47±170.84
Colitic EPCR-TgN	743.22 ± 47.66	858.85 ± 60.20
Control WT + murine APC	657.13 ± 41.21	968.44 ± 80.53
Colitic WT + murine APC	833.50 ± 149.13	914.42 ± 132.62
Control WT + APC mAb	712.98 ± 65.85	917.72 ± 97.73
Colitic WT + APC mAb	845.47 ± 120.02	1088.38 ± 144.32

* Colitic mice received 3 % DSS, while controls received water only.