Organ-Specific Protection Against Lipopolysaccharide-Induced Vascular Leak Is Dependent on the Endothelial Protein C Receptor

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Objective—To study the role of the endothelial protein C receptor (EPCR) in the modulation of susceptibility to inflammation-induced vascular leak in vivo.

Approach and Results—Genetically modified mice with low, <10% EPCR expression (EPCR<sub>low</sub>) and control mice were challenged with lipopolysaccharides in a mouse model of endotoxemia. Infrared fluorescence and quantification of albumin-bound Evans Blue in tissues and intravascular plasma volumes were used to assess plasma extravasation. Pair-wise analysis of EPCR<sub>low</sub> and control mice matched for sex, age, and weight allowed determination of EPCR-dependent vascular leak. Kidney, lung, and brain were the organs with highest discriminative increased Evans Blue accumulation in EPCR<sub>low</sub> versus control mice in response to lipopolysaccharides. Histology of kidney and lung confirmed the EPCR-specific pathology. In addition to severe kidney injury in response to lipopolysaccharides, EPCR<sub>low</sub> and anti-EPCR-treated wild-type mice suffered from enhanced albuminuria and profound renal hemorrhage versus controls. Intravascular volume loss at the same extent of weight loss in EPCR<sub>low</sub> mice compared with control mice provided proof that plasma leak was the predominant cause of Evans Blue tissue accumulation.

Conclusions—This study demonstrates an important protective role for EPCR in vivo against vascular leakage during inflammation and suggests that EPCR-dependent vascular protection is organ-specific. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: endothelial protein C receptor • endothelium • endotoxemia • inflammation • vascular leak

The endothelial protein C receptor (EPCR) is a predominant vascular cell receptor. EPCR binds both protein C (PC) zymogen circulating and the serine protease activated protein C (APC) with similar affinity, thereby localizing PC and APC to the cell surface. Although EPCR is considered a nonsignaling receptor because of its short (Arg-Arg-Cys) intracellular tail, it is increasingly recognized as a key receptor in the anticoagulant and cytoprotective PC pathways by facilitating (APC’s) interactions. Binding of PC to EPCR facilitates activation of PC to APC by the thrombin–thrombomodulin complex. APC acts as anticoagulant, and it also conveys cytoprotective functions that require EPCR and protease activated receptor 1 (PAR1), and include antinflammatory and antiapoptotic activities and endothelial barrier-stabilizing effects. The important contributions of the PC pathway are evident from the severe thrombotic and inflammatory complications in newborns with homozygous PC deficiency that typically presents as neonatal purpura fulminans with microvascular thromboembolism and necroses. In vivo murine endotoxemia and sepsis studies showed that a cytoprotective-selective APC mutant reduced mortality equivalent to wild-type APC, whereas an anticoagulant-selective APC mutant did not. As the mortality-reducing activity of APC in murine sepsis models required EPCR and PAR1, it suggested that the EPCR-mediated PAR1 cleavage by APC is likely a major contributing factor to mortality reduction in sepsis.

In addition to therapeutic cytoprotection, when APC is administered exogenously at high doses, there is mounting evidence that the PC pathway involving EPCR plays an important role for endogenous cytoprotection during inflammation. Endogenous depletion of PC or APC by inhibitory antibodies selectively blocking APC’s proteolytic or cytoprotective activities increased susceptibility to death in animal models of endotoxemia and sepsis (mice and baboons). Susceptibility to death during lipopolysaccharides (LPS)-induced mortality in mice with genetically low PC expression (10%–50% of normal) or low EPCR expression (<10% of normal; EPCR<sub>C</sub>) is significantly increased, whereas mice overexpressing EPCR are resistant to LPS-induced mortality. In humans, acquired PC deficiency during sepsis is associated with higher organ failure rates and higher mortality. PC depletion is thought to be caused by liver failure,
consumptive coagulopathy, and possibly by EPCR shedding into circulation.18 Serum concentrations of soluble EPCR in humans are elevated in sepsis and inflammatory autoimmune disorders consistent with the observations that genetic or acquired defects of the endogenous PC pathway exacerbate the negative effects of inflammatory disease and sepsis.18–20

To date, the mechanisms for EPCR-dependent protection against mortality during endoxemia and sepsis by APC are incompletely understood. According to the current paradigm, EPCR plays a central role by localizing APC to caveolin-1–rich microdomains and acting as a cofactor for APC-mediated PAR1 cleavage that results in activation of cytoprotective signaling cascades.4,7 However, the functions of EPCR in normal hemostasis and cytoprotection are likely more complex. For example, APC-mediated downregulation of monocyte procoagulant activity that is dependent on APC-ligation to apolipoprotein E receptor 2 required EPCR but not PAR1, whereas APC-protective effects that were dependent on CD11b/CD18 were found to be independent of EPCR.21,22 In vitro, the presence of EPCR helps to confer endothelial barrier integrity and is required for APC-mediated barrier protection that involves transactivation of the sphingosine-1-phosphate receptor 1.23–25

Because vascular barrier disruption, fluid extravasation, and rising interstitial tissue pressures are increasingly considered to be important pathophysiological determinants of organ failure and death,26 we studied the role of EPCR for vascular permeability in vivo using our novel infrared fluorescence (IRF) imaging methodology for the determination of Evans Blue (EB) plasma extravasation into organs.27 Application of this methodology to study the contribution of EPCR to vascular leakage, tissue edema, and organ injury provide unique insights into the role of EPCR in the pathophysiological induction of vascular leakage during endotoxemia. Low EPCR expression resulted in profound plasma tissue extravasation, lung injury, severe renal hemorrhage, and albuminuria. In addition, our data point toward an important role for EPCR in the organ-specific modulation of susceptibility to inflammatory disease, demonstrating that EPCR, hence the endogenous cytoprotective PC pathway, provides an important defense mechanism in vivo against vascular leak during inflammation and sepsis.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Determination of Lethality in C57Bl/6J Mice With LPS From Escherichia coli (serotype 0111:B4)

LPS derivatives from different bacteria, as well as different serotype preparations can vary widely in their potency to cause dose-dependent inflammatory reactions and death. Therefore, percentage lethality was determined for increasing doses of LPS from E. coli (0111:B4) in C57Bl/6J mice to relate degrees of vascular permeability to lethality rates in subsequent experiments. Females (n=186) and males (n=95) were injected with 4 to 5 dose levels of LPS ranging between 2 and 20 mg/kg. Seven-day lethality for both sexes was recorded and expressed in lethality doses (LD) for each dose level of LPS (Figure 1). Because male C57Bl/6J mice were more resistant to LPS than female C57Bl/6J mice, sex-specific dosing for

Figure 1. Sex-specific lipopolysaccharide (LPS)-induced mortality rates in C57Bl/6J mice. Sex-specific survival curves were constructed by injection of increasing doses of LPS intraperitoneal in female C57Bl/6J mice (n=186; 30–44 mice per group) or male C57Bl/6J mice (n=95; 16–23 mice per group). Lethality increased with increasing doses of LPS and was clearly different between (A) female and (B) male C57Bl/6J mice. Seven-day mortality rates for the various doses of LPS were assessed by Kaplan-Meier log-rank test and plotted against the LPS dose to determine the LD50 for (C) female and (D) male C57Bl/6J mice.
LPS injection was used to achieve the same percentage lethality for males or females in all subsequent experiments (male mice: LD25 [5.8 mg/kg], LD40 [8.9 mg/kg], and LD50 [10.8 mg/kg]; and female mice: LD25 [3.7 mg/kg], LD40 [5.2 mg/kg], and LD50 [6.9 mg/kg]). To ensure that an inflammatory response to LPS was present even when low doses of LPS were injected, weight loss as a surrogate marker for sickness was determined in C57Bl/6J mice at increasing doses of LPS, 18 hours after intraperitoneal injection. Weight loss was similar at all dose levels of LPS (LD10–LD80) and was present even if mice were not visibly sick. Weight loss was specific to treatment with LPS, as it was not observed in control animals injected with similar amounts of denatured protein or rat IgG (Figure I in the online-only Data Supplement).

EB Dye Accumulation in Organs of EPCRlow Mice
To determine the role of EPCR in the modulation of vascular integrity, EPCRlow and control mice that were matched for age, weight, and sex were injected as pairs with intraperitoneal saline (baseline; n=12), LPS at LD25 (n=8), LD40 (n=14), or LD50 (n=4). Because LPS doses exceeding LD60 to LD70 caused high mortality (∼70%) during the first 2 days, with animals too sick for vascular leakage experimentation, we limited our vascular permeability studies to LD25 (low-dose LPS) and LD40–50 (medium-dose LPS). Organ pairs of EPCRlow and control mice were compared directly and results expressed as relative fold increase of IRF in the organ of an EPCRlow mouse compared with its matched control (n=119; organ pairs of liver, spleen, lung, kidney, heart, and brain).

At baseline (saline injection), EB accumulation in organs of EPCRlow was similar to that of control mice (mean fold increase 1.1), indicating that low EPCR expression did not result in loss of vascular barrier function during normal physiology, or that compensatory mechanisms are sufficient to overcome the deleterious effect of low EPCR expression (Figure II in the online-only Data Supplement). However, challenging vascular barrier function by administration of LPS resulted in increasing EB accumulation in organs of EPCRlow compared with control mice. At low-dose LPS, mean EB accumulation in organs of EPCRlow compared with control mice was increased 1.6-fold (P=0.08) from baseline (saline) and 2.0-fold (P<0.0006) at a medium dose of LPS (Figure II in the online-only Data Supplement). Methodology analyzing organ pairs of matched EPCRlow and control mice affords certainty that the increased EB accumulation reflects only EPCR-dependent effect and not LPS-induced changes that are similar in EPCRlow and control mice. Furthermore, at a medium dose of LPS, 80% (P=0.006) of organ pairs showed significantly increased EB accumulation from baseline in EPCRlow mice compared with control mice (Figure II in the online-only Data Supplement). To determine in which organ systems vascular barrier function was most affected by low EPCR expression, individual organ systems were compared (Figure 2). Medium-dose LPS-induced EB accumulation in EPCRlow mice was significantly increased in the brain (3.4-fold increase; confidence interval [CI], 1.4–5.5), followed by kidney, lung (both 2-fold increase; CI, 1.2–2.8), and spleen (1.5-fold increase; CI, 1.1–1.9). Remarkably, no EPCR-dependent increase of EB accumulation was observed in the liver after medium-dose LPS, suggesting that changes in vascular barrier function in the liver induced by LPS are not exacerbated by low EPCR expression (Figure 2).

Figure 2. Organ-specific sensitivity to lipopolysaccharides (LPS)-induced Evans Blue (EB) accumulation in mice expressing <10% endothelial protein C receptor (EPCRlow) compared with control mice. EPCRlow and matched wild-type controls were injected with (A) intraperitoneal saline (n=12; 6 pairs), (B) low-dose LPS (n=8; 4 pairs), or (C) medium-dose LPS (n=18; 9 pairs). Organs were harvested 18 hours later, 30 minutes after intravenous injection of EB. EB accumulation was expressed as the EB fluorescence at 700 nm in relation to organ weight and peak total plasma infrared fluorescence (IRF). Each single data point represents the relative difference of EB dye accumulation in the organ of an EPCRlow mouse compared with its matched control mouse (y axis; 1, no difference). Wilcoxon matched-pairs signed rank test was used for analyses of differences. Error bars represent SEM. NS denotes no significant difference between EPCRlow and control mice.
Increased Intravascular Volume Contraction in EPCR<sub>low</sub> Mice After LPS

To investigate whether EB dye accumulation in organs of EPCR<sub>low</sub> mice was a result of direct plasma extravasation into tissues as opposed to blood pooling or dehydration, the degree of intravascular plasma contraction in relation to weight loss was determined. Compared with control mice (n=9), where hematocrits before and after LPS (medium-dose) were not different (mean hematocrit 44.5% versus 45.6%), hematocrits in EPCR<sub>low</sub> mice (n=9) rose significantly from a mean of 47.3% to 57.7% (P<0.0005; Figure 3). No significant loss of intravascular plasma volume was present after injection of low-dose LPS (data not shown). Remarkably, in 4 of the 9 EPCR<sub>low</sub> mice, hematocrits exceeded 60%. In parallel, average total plasma volumes decreased significantly in EPCR<sub>low</sub> mice from 34.3 mL/kg before LPS to 27.5 mL/kg after LPS (P<0.0004). In contrast, plasma volumes remained similar in control mice (36.1 mL/kg versus 35.3 mL/kg). Percentage weight loss after LPS was similar for EPCR<sub>low</sub> and control mice (6.5% versus 7.5%; P=0.6; Figure 3). Taken together, the significantly decreased intravascular plasma volumes in EPCR<sub>low</sub> mice after LPS at the same degree of weight loss compared with wild-type mice is diagnostic of plasma extravasation into tissues. Neither dehydration nor blood pooling could have been important mechanisms of EB tissue accumulation, as dehydration would have been associated with increased weight loss, and blood pooling would have not affected the hematocrit. Thus, low EPCR expression results in a significant increase in hematocrits and contraction of intravascular plasma volumes after LPS challenge.

EPCR<sub>low</sub> Mice Suffered From Severe Renal Injury and Albuminuria

To define the pathology associated with EPCR-dependent plasma extravasation in response to LPS, histology was performed on kidneys, lung, and liver. Kidneys of EPCR<sub>low</sub> and matched control mice receiving LPS (medium-dose) or saline (baseline) were harvested and processed for histology immediately after IRF imaging. Compared with control mice, kidneys of EPCR<sub>low</sub> mice were found to have significantly increased EB dye accumulation after LPS (Figure 2) and demonstrated pronounced renal injury, which was absent in wild-type mice (Figure 4). No differences in organ histology were present at baseline (data not shown). After LPS, kidneys of EPCR<sub>low</sub> mice were visually swollen and had a significantly increased kidney-to-body weight ratio compared with control mice (Figure 4). Histological analysis revealed profound renal hemorrhage at the corticomedullary junction in kidneys of EPCR<sub>low</sub> mice that was not obvious in kidneys of control mice receiving LPS (Figure 4). In addition, EPCR<sub>low</sub> mice had significantly increased albuminuria (2.6-fold; P=0.03) compared with controls after LPS, whereas no difference was observed at baseline (Figure 5). Albuminuria, expressed as IRF/mL urine, was assessed at baseline and after LPS (medium-dose) by determination of EB accumulation in harvested bladders in relation to bladder weight (=urine volume) and peak total plasma IRF. As albuminuria under physiological conditions is negligible, the appearance of albumin in urine after LPS is therefore pathognomonic of glomerular permeability.

In addition to kidneys, also lungs showed increased EPCR-dependent EB accumulation in response to LPS (Figure 2). Histological analysis was consistent with more pronounced lung edema and injury to lungs of EPCR<sub>low</sub> mice compared with controls. Lungs of EPCR<sub>low</sub> mice showed evidence of septal thickening with accumulation of polymorphonuclear leukocytes, karyorrhectic debris, and increased alveolar fibrillar materials that were not obvious in lungs of control mice receiving LPS (Figure 6).

Remarkably, in contrast to kidney and lung, no EPCR-dependent differences in relative EB accumulation were observed in the liver after LPS. Recently, we found that in C57Bl/6J (control) mice, areas of LPS-induced EB dye extravasation in the liver corresponded to focal areas of liver necrosis. Histological analysis of livers of EPCR<sub>low</sub> mice injected with LPS (medium-dose) showed similar findings of focal areas of liver necrosis corresponding to areas of EB dye extravasation (Figure III in the online-only Data Supplement). Moreover, at baseline (saline injection), livers of EPCR<sub>low</sub> and control mice appeared similar (data not shown). Thus, histological findings of relatively similar liver pathology in EPCR<sub>low</sub> and control mice after LPS are consistent with the observed lack of any EPCR-dependent difference in EB accumulation in the liver. Notwithstanding these similarities, histology of the liver did reveal a major difference between EPCR<sub>low</sub> and control mice after LPS. Whereas the necrotic areas of the liver were infiltrated with polymorphic mononuclear cells in control mice, infiltration of corresponding necrotic areas with polymorphic mononuclear cells in livers of EPCR<sub>low</sub> mice was notably less (Figure III in the online-only Data Supplement).
EB Dye Accumulation in Organs of Mice Treated With Antimouse EPCR Antibody

We next determined whether EPCR-dependent sensitivity to LPS-induced vascular leak could also be revealed in C57BL/6J wild-type mice using a blocking antibody against mouse EPCR (rcr-16).28,29 Wild-type mice that were matched for age, weight, and sex received rcr-16 or vehicle control (saline) 2 hours before LPS at LD50 (n=8 pairs; 16 mice). Organ pairs of antibody-treated and control mice were compared directly and results expressed as relative fold increase of IRF in the organ of an antibody-treated mouse compared with its matched control. As EPCRlow mice, wild-type mice treated with blocking EPCR antibodies demonstrated increased EB accumulation in organs compared with control mice. The pattern of organ-specific EPCR-dependent sensitivity to LPS-induced vascular leak was attenuated, but generally similar to that observed in EPCRlow mice (Figure IV in the online-only Data Supplement). Notably, significant albuminuria and EB accumulation were found in kidneys of mice treated with blocking EPCR antibody compared with saline. A 2.3-fold (CI, 1.4–3.5) increase of EB accumulation in kidneys of mice treated with EPCR-blocking antibody compared with saline was present, and albuminuria was 3-fold (CI, 1.6–4.4) increased, respectively. These results were comparable with the renal findings in EPCRlow mice. Thus, blocking EPCR function in wild-type mice or using EPCRlow mice identified the kidney as an organ with high-sensitivity to EPCR-dependent protection against LPS-induced vascular leak. This corroborates that the observed discriminative effects between EPCRlow and control mice in the kidney involved EPCR-dependent mechanisms and were not the result of a developmental defect caused by low EPCR expression or an unknown genotype difference between EPCRlow and wild-type mice.

Discussion

This study emphasizes the important protective role EPCR plays in vivo in vascular barrier stabilization during inflammation.23,24 Although EPCR was demonstrated to be required for endothelial barrier protection in vitro, this function of EPCR has not been previously established in vivo. The role of EPCR for vascular leakage in vivo was determined using our recently developed IRF technology for quantification of EB accumulation in organs.27 Pair-wise analysis of EPCRlow and control mice that were matched for sex, age, and weight allowed for the specific detection of EPCR-dependent differences in vascular barrier function in an LPS-induced mouse model of endotoxemia. Proof of plasma leak as the predominant cause of EB tissue accumulation was provided by demonstration of profound intravascular volume loss at the same extent of weight loss in EPCRlow mice compared with wild-type mice, thus excluding dehydration for the volume contraction, or blood pooling for accumulation of EB dye in central organs.
Kidney and lung were the solid organs that showed the highest discriminative EB accumulation between EPCRlow and control mice in response to LPS, suggesting that EPCR plays an important role in maintaining vascular integrity during inflammation, especially in these organs. The increased susceptibility to LPS-induced vascular deterioration because of low EPCR expression (<10%) was supported by histological findings of vascular permeability, such as renal hemorrhage and pulmonary alveolar edema. In contrast, at the LPS dose that induced EPCR-dependent vascular deterioration in kidneys and lungs, focal necroses were the major pathological findings in the liver. No EPCR-dependent differences in susceptibility to focal necrosis were found, as livers of both EPCRlow and control mice were affected to a similar extent. These findings coincided with a similar degree of EB dye accumulation in the livers of these mice, supporting the absence of a clear role of EPCR for modulating vascular integrity in the liver in endotoxemia. In summary, these data suggest that the relative importance of EPCR-dependent protection from vascular leak is organ-specific.

The organ system most severely affected by low EPCR expression was the kidney, as evident from both the EPCRlow mice and wild-type mice treated with blocking EPCR antibodies. In addition to significant EB dye accumulation in the kidneys of EPCRlow mice compared with control mice, EPCRlow mice suffered from severe kidney injury in response to LPS that was not obvious in control mice. Findings consisted of kidney swelling, albuminuria, and profound renal hemorrhage at the corticomedullary junction, indicative of the most extreme expression of vascular breach. These EPCR-mediated effects on renal integrity in endotoxemia are consistent with previously reported roles of the PC pathway for renal protection in vitro, diabetic nephropathy injury models in vivo, and patients with chronic renal failure on hemodialysis.30–33

In the brain, significantly increased EB dye accumulation was detected in EPCRlow compared with control mice after LPS. These findings provide further evidence that EPCR may be important to prevent blood–brain barrier disruption during inflammation, extending findings where mice overexpressing EPCR were protected against brain edema in a model of venous sinus thrombosis.35 EPCR is present on vascular endothelial cells in the central nervous system, and facilitates the transport of APR across the blood–brain barrier to provide cytoprotective activities on neurons and microglia cells.36,37 Although encephalopathy is a well-recognized and alarming complication of sepsis associated with poor outcomes, the pathophysiological processes underlying inflammatory blood–brain barrier breakdown remain poorly understood.38 Our results indicate that low EPCR expression enhanced susceptibility to LPS-induced blood–brain barrier breakdown, providing new potential leads to elucidate the mechanisms leading to sepsis-associated encephalopathy. Furthermore, these new insights suggest the blood–brain barrier (and the brain) as a previously underappreciated target for the endogenous PC pathway in sepsis, consistent with the well-documented neuroprotective effects of pharmacological APC applications in ischemic stroke.39

As the vascular barrier integrity in endotoxemia is EPCR-dependent, we speculate that the molecular pathway using EPCR for barrier protection is the endogenous PC pathway. We base this assumption on our understanding of the well-described EPCR-dependent activation of PC- and EPCR-dependent cytoprotective activities of pharmacological APC on cells and tissues.6,12,40 Additional compelling support for the protective role of the endogenous PC pathway is also provided by the results from previous animal studies in sepsis or endotoxemia, where modulation of important players of the endogenous PC system, such as PC expression, EPCR expression, or antibody blockade of (A)PC or EPCR, all similarly modulate survival.13,15,16 For instance, pharmacological APC administration reduced LPS-induced mortality in wild-type mice, but not in EPCRlow mice.10,31 In addition, rescued EPCR-deficient mice had a prothrombotic phenotype and expressed lower levels of APC, whereas mice overexpressing EPCR demonstrated higher levels of circulating APC after thrombin infusion.16,41 However, additional or alternative mechanisms remain possible, especially because EPCR is expressed on vascular endothelial and on blood and immune cells. Although our results demonstrating profound organ-specific vascular leakage are consistent with low expression of EPCR on vascular endothelial cells, additional functions of EPCR mediated by blood and immune cells may contribute to host defense and antiinflammatory effects. Our observation of diminished polymorphic mononuclear infiltrate in livers of EPCRlow but not control mice after LPS underlines
Figure 6. Mice expressing <10% endothelial protein C receptor (EPCRlow) suffered from increased lung permeability and injury after lipopolysaccharides (LPS). Shown are 2 representative experiments where EPCRlow and matched control mice received medium-dose LPS (n=8; 4 pairs). Lungs were harvested 18 hours later, 30 minutes after intravenous Evans Blue (EB; 25 mg/kg). A, Photographs demonstrated increased EB color in lungs of EPCRlow compared with wild-type mice. B, EB was quantified by infrared fluorescence (IRF) spectroscopy for each pair of mice in relation to organ wet weight and peak total plasma IRF. C, Odyssey scans at 700 nm of the lungs. D, Histology (H/E) of lungs revealed increased septal thickening with accumulation of polymorphonuclear leukocytes and karyorhectic debris (black arrow), and increased alveolar fibrillar material (blue arrow) consistent with more pronounced lung edema in EPCRlow mice.

In summary, our results demonstrate that EPCR is required to maintain vascular integrity and prevent tissue edema in vivo during endotoxemia. Responses to inflammation seem to be organ-specific, with kidneys, lungs, and brains strongly affected by EPCR-deficiency. Tissue edema and vascular leak in sepsis and inflammation are increasingly recognized as a deleterious entity that predisposes to organ failure and mortality. Understanding extravasation, tissue edema, and vascular barrier stabilization on a molecular level has just begun, and may open new avenues for life-saving barrier-protective agents in sepsis, such as strategies aimed at improving or retaining EPCR expression on vascular endothelial and blood cells during inflammation.

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Disclosures
None.

References
The study demonstrates that the endothelial protein C receptor (EPCR), important for protein C (PC) activation and activated protein C (APC) cytoprotective effects on cells, is required to maintain organ-specific vascular integrity during endotoxemia. Using highly sensitive novel infrared fluorescence imaging techniques and measurements of intravascular volume depletion EPCR-dependent susceptibility to lipopolysaccharides (LPS)-induced vascular leakage was determined in various organs. The kidneys, lungs, and brains of mice expressing <10% EPCR (EPCRlow) mice or wild-type mice treated with blocking anti-EPCR antibodies were shown to be particularly vulnerable to endotoxemia-induced vascular leakage compared with wild-type control mice. This study supports the important protective role for the endogenous cytoprotective PC pathway against vascular leakage during inflammation, and demonstrates that EPCR-dependent vascular protection is organ-specific. Because vascular leakage is increasingly recognized as deleterious within the pathophysiology of sepsis, strategies aimed at improving or retaining EPCR expression on vascular endothelial and blood cells during inflammation may prove to be beneficial.
Material and Methods

Animals

All animal protocols were approved by the institutional animal and care committee of The Scripps Research Institute. EPCR$^{δ/δ}$ (a.k.a. EPCR$^{low}$) mice that express <10% EPCR (generously provided by Dr. Francis Castellino, University of Notre Dame, Notre Dame, IN) were used (C57Bl background, > 10 backcrosses) since complete genetic deficiency of EPCR in mice is embryonically lethal.\textsuperscript{1, 2} Control wild-type C57Bl/6J were from The Scripps Research Institute Breeding facility. In all experiments, each EPCR$^{low}$ mouse was matched for weight ($\leq +/\text{-}5\%$), age ($\leq +/\text{-} 7$ days) and gender with a C57Bl/6J wild-type mouse prior to the experiment and results were analyzed by direct comparison of the pairs. Alternatively, pairs of C57Bl/6J wild-type mice receiving saline were matched for weight ($\leq +/\text{-}5\%$), age ($\leq +/\text{-} 7$ days) and gender with mice receiving 50 μg of the EPCR blocking rcr-16 rat anti-mouse EPCR antibody (generously provided by Dr. Kenji Fukudome, Saga Medical School, Saga, Japan and Dr. Ramon Montes, University of Navarra, Pamplona, Spain).

LPS-injections and determination of lethality doses (LD)

Mice, 6-10 weeks old, were injected intraperitoneally (i.p) with LPS (E.coli; serotype 0111:B4; Calbiochem) solubilized in 0.9% sterile sodium chloride for injection (Hospira Inc) by sonication and vortexing. C57Bl/6J mice of both genders were injected with increasing doses of LPS (2-20 mg/kg). Seven-day mortality rates at each dose level were recorded and percent mortality at every dose level was expressed as gender-specific LD. In all further experiments, gender-specific LD and LPS i.p. concentration refer to those obtained in C57Bl/6J wild-type mice. Mouse weights were determined on a digital pocket scale (Fast Weigh Scales M-500) at 18 hours after i.p. LPS injection as a surrogate marker for sickness. Control groups of C57Bl/6J mice were injected with sterile filtered polyclonal rat IgG (Pierce, 10 mg/kg) or denatured protein mouse IgG obtained by boiling for 10 min to demonstrate that weight loss is specific to LPS and not to injection, experimental handling, or foreign material.

Determination of EB in organs and plasma by infrared (IRF) imaging

IRF imaging for EB content in tissues and plasma was performed as described.\textsuperscript{3} Briefly, 18 hours after LPS injection, EB (Sigma) at 25 mg/kg (0.9% sterile sodium chloride for injection (Hospira Inc)) was injected intravenously (i.v.) by lateral tail vein injection. Peak plasma values of EB were determined 2 minutes later in blood collected retro-orbitally into heparin-coated microcapillaries (75 uL; Fisher Scientific) as described.\textsuperscript{3}
At 30 minutes, organs were harvested after severing the vena cava below the renal vessels and organs were flushed by intracardiac instillation of 40 mL saline. EB IRF was performed using the Odyssey infrared imager (LI-COR) with Application software version 3.0 using the 700 channel (excitation by solid-state diode laser at 685 nm and emission 700-750 nm) with the focal plane of the microscope head set at 1 mm. Freshly harvested organs were weighed and, dependent on organ size, placed on the inner surface of lids from 6-, 12- or 24-tissue culture plates (Costar®, Corning Inc). Small round or cubic shaped organs (heart, spleen, kidney) were cut in half and pressed gently onto the plastic surface. Each organ system was scanned separately using laser intensities that provided an optimal linear range of fluorescence intensities for each organ system. After scanning, organ shapes were encircled by the automated drawing tool provided with LI-COR analysis software and fluorescence intensity captured over the encircled organ area was recorded as raw fluorescence intensities (designated RFI per manufacturer). To correct for the complicated volume shifts that might have occurred during the course of LPS injury, RFI were multiplied by fresh organ weight and divided by peak plasma RFI as described. Results were expressed as the relative-fold increase of EB fluorescence in organs of an EPCR<sub>low</sub> mice compared to wild-type mice matched by age, gender and weight.

**Determination of intravascular plasma volumes**

Intravascular plasma volume in EPCR<sub>low</sub> as well as control mice before and after LPS was calculated under the assumption that total blood volume equals 65 ml/kg. In general, blood volumes in mammals and man are fairly consistent and range between 60-75 ml/kg and are reported to be < 70 ml/kg for mice. Therefore, 65 mL/kg seemed a reasonable supposition for all calculations. Since the total blood volume is divided in plasma and red cell volume, plasma volume can be calculated based on spun hematocrits (packed red cell volume) as a percentage of total blood volume multiplied by actual body weight [(100-hematocrit)/100x65ml/kg]. Plasma volumes were determined one week prior to each experiment (baseline) as well as 18 h after LPS administration. Blood was drawn into heparin-coated microcapillaries (Fisher Scientific), sealed with Critoseal® (Leica Microsystems), spun in a hematocrit centrifuge (International Equipment Company; New Jersey) and, hematocrits were determined by a integrated circular reader.
Histology
Following IRF spectroscopy, fresh tissues were placed in ZnSO$_4$-buffered 10% formalin. Formalin-fixed tissues were processed routinely, embedded in paraffin, sectioned at 3 micron, and stained with hematoxylin and eosin (H/E) and/or Carstairs stains.

Statistical analysis
Student’s t-test was used for parametric data. For non-parametric data Wilcoxon matched-pairs signed rank test or Mann Whitney test was used to assess statistical significance as appropriate. Depending on the test, p-values of ≤ 0.05 and/or 95% confidence intervals (CI) provided statistical significance. Survival was plotted using Kaplan-Meier survival curves.

Reference List


Supplemental Figure I. LPS-induced weight loss. Female C57Bl/6J mice received increasing doses of LPS i.p. (2-12 mg/kg; LD 10-80). Weights were determined prior to and 18 hours after injection. Weight loss was significant at all dose levels of LPS, even at LD10. As a negative control, groups of C57Bl/6J mice were also injected with similar amounts of sterile polyclonal rat IgG or sterile denatured protein (mouse IgG). Student’s t-test was used for analysis of differences. Error bars represent standard error of the mean. * Indicates statistical significance (p ≤ 0.05). Denat.Prot denotes denatured protein.
Supplemental Figure II. EPCR-dependent EB accumulation in organs upon LPS challenge. EPCR\textsuperscript{low} mice and matched wild-type controls were injected with i.p saline (n=12), low dose LPS (n=8) or medium dose LPS (n=18). Organs were harvested 18 h later, 30’ after i.v. injection of EB (25 mg/kg) and EB fluorescence in organs was quantified using the Odyssey Infrared Imager and expressed as IRF based on organ weight and peak total plasma fluorescence. Each single data point represents the relative difference of IRF in the organ of an EPCR\textsuperscript{low} mouse compared to its matched control mouse (Y-axis: 1= no difference). Mann-Whitney test for non-parametric data was used for statistical significance (* denotes p<0.01). Error bars represent SEM.

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<tr>
<td>95% CI</td>
<td>0.9 - 1.2</td>
<td>0.9 - 2.3</td>
<td>1.6 - 2.3</td>
</tr>
<tr>
<td>% EPCR\textsuperscript{low} organs with increased EB dye compared to wild-type</td>
<td>17/36 = 47%</td>
<td>15/29 = 51%</td>
<td>43/54 = 80%</td>
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Supplemental Figure III. LPS-induced liver pathology was similar for EPCR<sub>low</sub> and control mice. Shown is a representative experiment where EPCR<sub>low</sub> mice and matched controls received i.p. medium dose LPS. Livers were harvested 18 h later, 30’ after i.v. EB. (A) Odyssey scans at 700 nm of the livers. (B) EB was quantified by IRF spectroscopy for each pair of mice in relation to organ wet weight and peak total plasma IRF. (C) Histology (H/E) of livers revealed areas of focal necrosis with infiltration of polymorphic mononuclear cells (blue rectangle).
Supplemental Figure IV. Organ-specific sensitivity to LPS-induced EB accumulation in wild-type mice injected with anti-mouse EPCR antibody compared to control mice injected with saline. Matched wild-type mice were injected with EPCR blocking antibody rcr-16 or saline 2 hours prior to i.p medium dose LPS (n=16; 8 pairs). Organs were harvested 18 h later, 30' after i.v. injection of EB. EB accumulation was expressed as the EB fluorescence at 700 nm in relation to organ weight and peak total plasma IRF. A) Each single data point represents the relative difference of EB dye accumulation in the organ of a mouse injected with anti-mouse EPCR antibody compared to its matched control mouse injected with saline (Y-axis: 1= no difference). B) Kidney EB quantification per group displayed as absolute values of EB IRF. C) Shown is a representative example of IRF in kidneys in a pair of matched mice on the LI-COR Odyssey. D) Relative change in albuminuria between pairs of mice injected with anti-mouse EPCR antibody or saline. Each single data point represents the relative difference of EB IRF in urine of a mouse treated with anti-mouse EPCR antibody compared to its matched control mouse (Y-axis: 1= no difference). Wilcoxon matched-pairs signed rank test was used for analyses of differences. * denotes statistical significance (p-value < 0.01). Error bars represent SEM. NS denotes no significant difference between mice injected with anti-mouse EPCR antibody and saline.