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Thrombin Augments LPS-Induced Human Endometrial Endothelial Cell Inflammation via PAR1 Activation

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Abstract

Problem—Risk factors for preterm birth include placental abruption, giving rise to excessive decidual thrombin, and intrauterine bacterial infection. Human endometrial endothelial cells (HEECs) express Toll-like receptors (TLRs), and infection-derived agonists trigger HEECs to generate specific inflammatory responses. Since thrombin, in addition to inducing coagulation, can contribute to inflammation, its effect on HEEC inflammatory responses to the TLR4 agonist, bacterial lipopolysaccharide (LPS), was investigated.

Method of Study—HEECs were pretreated with or without thrombin or specific protease-activated receptor (PAR) agonists, followed by treatment with or without LPS. Supernatants were measured for cytokines and chemokines by ELISA and multiplex analysis.

Results—Thrombin significantly and synergistically augmented LPS-induced HEEC secretion of interleukin (IL)-6, IL-8, granulocyte colony stimulating factor (G-CSF), and growth regulated oncogene-alpha (GRO-α); and significantly augmented monocyte chemotactic protein (MCP)-1, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF) secretion additively. Similar to thrombin, a PAR1 agonist synergistically augmented the LPS-induced HEEC secretion of inflammatory IL-6, IL-8, G-CSF, and GRO-α.

Conclusion—Thrombin, via PAR1 activation, synergistically augments LPS-induced HEEC production of chemokines involved in immune cell recruitment and survival, suggesting a mechanism by which intrauterine abruption and bacterial infection may together be associated with an aggravated uterine inflammatory response.

Keywords

Endothelial; Infection; Inflammation; Pregnancy; Thrombin

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Introduction

Preterm birth affects 9.6% of US births [1] and is the leading cause of morbidity and mortality in newborns [2]. Preterm infants have increased rates of respiratory illnesses, feeding difficulties, and neurodevelopmental issues. Every year, over $26 billion are spent in the US on healthcare costs related to preterm birth [3] with no significant decrease in mortality from 2000 to 2010 [4]. Our understanding of preterm birth is still evolving and our ability to prevent preterm birth remains limited. Therefore, a better understanding of the molecular basis of preterm birth is needed to predict and prevent this disorder.

Elucidation of the mechanism(s) underlying preterm birth is complicated by its heterogeneous antecedents including bacterial infection, placental abruption, cervical incompetence, and disparate risk factors including prior preterm birth, smoking, prior cervical surgery, and low pre-pregnancy weight [5]. However, a likely common pathway leading to preterm birth is inflammation at the maternal-fetal interface [6–8]. Since bacterial infection and abruption are both important risk factors for preterm birth [9], this study sought to determine the relationship between decidual infection and hemorrhage at the molecular level and how this might impact pregnancy outcome.

The convergence of placental abruption and bacterial infection is commonly seen in the setting of preterm premature rupture of membranes (PPROM) [10]. In this clinical scenario, chronic placental abruption and indolent inflammation can lead to preterm labor. In many cases of PPROM, women do not display overt clinical signs of infection; confirmation of infection and inflammatory changes is often noted upon pathologic analysis of the placenta and fetal membranes. Similarly, the patient may not acutely hemorrhage, but may have small but persistent amounts of vaginal bleeding, suggesting a chronic placental abruption [9].

During placental abruption, the decidua is exposed to blood borne clotting factors to generate high levels of thrombin [11], which can act directly on the endothelium [12]. Thrombin is the main effector protease of the coagulation cascade that converts fibrinogen into fibrin, facilitating clot formation. However, thrombin can mediate a number of inflammatory effects by binding to the protease-activated receptors (PARs), PAR1, PAR3 and PAR4, which stimulate endothelial cells and other cell types to express cytokines, chemokines and other inflammatory mediators [13, 14]. Similarly, inflammation can influence the coagulation process [14].

Intrauterine bacterial infections can present as an ascending infection, a systemic infection originally stemming from a distant site and now circulating in the maternal bloodstream, or a descending infection from another intra-abdominal organ [8]. For systemic infections, the endothelium is the first tissue exposed to invasive pathogens in the bloodstream. The endothelial response to infection or tissue injury is to become activated to recruit immune cells and express adhesion molecules that allow their extravasation into tissues for host defense [15]. Thus, this study questioned the impact a systemic bacterial infection would have on the uterine endothelium in the context of excess thrombin production arising from abruption.

We previously demonstrated that human endometrial endothelial cells (HEECs) express Toll-like receptors (TLRs) and following their activation by infection-derived agonists mediate specific innate immune inflammatory responses [16, 17]. When HEECs, expressing TLR4 [18], are activated by bacterial LPS, they secrete elevated levels of inflammatory cytokines and chemokines [17].

Given that thrombin is a critical component of the inflammatory and coagulation cascades, and that HEECs express a specific inflammatory profile in response to bacterial LPS [17], we hypothesized that thrombin, as itself a mediator of inflammation, may act to strengthen this pro-inflammatory response of HEECs to bacterial stimulation by modulating the TLR4 pathway through PAR activation.

Materials and Methods

Human Endometrial Endothelial Cell (HEEC) Culture and Treatments

The HEECs used in this study were isolated, immortalized, and characterized as previously described [18–20]. Previous studies have demonstrated that these cells express the same factors and cytokines as the intact physiological endothelium [19, 20]. Cell culture was performed as previously described [16, 20]. For treatment experiments, HEECs were plated in 60mm tissue culture plates coated with 2% gelatin and grown until 70% confluent. Media was then replaced with serum-free OptiMEM (Gibco-Invitrogen; Grand Island, NY) with or without physiological concentrations of thrombin (Sigma-Aldrich, St. Louis, MO; 0.25 U/ml) and incubated for 1 hr. Without changing the media, cells were then treated with or without LPS (isolated from *Escherichia coli* 0111:B4; Sigma-Aldrich) at 1μg/ml, and the cells incubated for an additional 24 or 48 hrs. In subsequent treatment experiments, in place of thrombin, HEECs were treated with either a specific PAR1 agonist (TFLLR-NH2); a specific PAR2 agonist (SLIGKV-NH2); or a specific PAR4 agonist (AYPGKF-NH2) at 1mM (Sigma-Aldrich). After each time point, cell-free culture supernatants were collected and stored at −80°C. Cells were lysed for either RNA or protein isolation.

Cytokine and Chemokine Analysis

Supernatants were measured for IL-8 by ELISA (Assay Designs/Enzo Life Sciences; Farmingdale, NY). Supernatants were also measured for levels of interleukin (IL) 1β, IL-6, IL-10, IL-12, IL-17, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFNγ), Monocyte chemotactic protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1a (MIP-1α/CCL3), MIP-1β (CCL4), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), growth regulated oncogene-alpha (GRO-α, and interferon gamma-induced protein 10RO-(IP-10/CXCL10) by multiplex analysis (BioRad; Hercules, CA) as previously described [21].

Quantitative Real Time RT-PCR

Quantitative RT-PCR was performed as previously described [22, 23]. Briefly, total RNA was extracted from cells, isolated, and reverse transcribed, after which quantitative real-time

PCR was performed for TLR4 (Forward: 5′-CAGAGTTTCCTGCAATGGATCA-3′; Reverse: 5′-GCTTATCTGAAGGTGTTGCACAT-3′); PAR1 (Forward: 5′-AGTAGGCTATTCCTGAGAGCTGCAT-3′; Reverse: 5′-ATGGCCCTGGCATGTGTCT-3′); PAR2 (Forward: 5′-GGCAGGTGAGAGGCTGACTT-3′; Reverse: 5′-CGCCACCGATTCGAAACT-3′); PAR3 (Forward: 5′-TTGTGATTTTTACCATTTGCTTTG-3′; Reverse: 5′-TTTGTAAGGTAAGCAGTGGAGTGA-3′); PAR4 (Forward: 5′-GGGTCCCTTCCCCCACTT-3′; Reverse: 5′-GACAGTTGTAACAACCCTATTTCCAAA-3′); and GAPDH (Forward: 5′-CAGCCTCCCGCTTCGCTCTC-3′; Reverse: 5′-CCAGGCGCCCAATACGACCA-3′). Cycle threshold (CT) values were normalized to GAPDH and then presented as either relative abundance or fold change relative to the no treatment (NT) control.

Western Blot Analysis

HEEC protein was isolated and analyzed by Western blot as previously described [24]. Membranes were probed for SOCS1 and SOCS3 using antibodies from Cell Signaling (Danvers, MA; Cat #3950 (1:500) and #2923 (1:1000)). Hsp90 (H-114; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:1000 dilution as internal control to validate the amount of protein loaded onto the gels. Images were recorded and semi-quantitative densitometry performed using the Gel Logic 100 system and Carestream software (Carestream Molecular Imaging, Woodbridge, CT).

Statistical Analysis

Experiments were performed at least three times and data presented as mean ± SEM. Statistical significance ($p<0.05$) was determined by analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons using Prism software (Graphpad Software Inc, La Jolla, CA).

Results

Thrombin augments LPS-induced HEEC inflammation

We previously demonstrated that treatment of HEECs with LPS induces the secretion of inflammatory IL-8, IL-6 and G-CSF [17]. In this study, we sought to expand our knowledge of HEEC-derived factors regulated by LPS and to investigate if this response was modified when cells were pre-treated with thrombin. We first evaluated IL-8 secretion after 24 and 48hrs. As shown in Figure 1, after 24 hours, IL-8 secretion was not significantly elevated in HEECs treated with thrombin alone compared to the no treatment (NT) control. However, at the same time point, IL-8 levels were significantly increased in HEECs treated with LPS alone compared to the NT control. Furthermore, HEECs treated with both thrombin and LPS exhibited a synergistic 1.89 ± 0.21 fold increase in IL-8 secretion compared to LPS alone. After 48 hours, IL-8 secretion was significantly elevated in HEECs treated with either thrombin alone or LPS alone compared to the NT control (Figure 1). Moreover, HEECs treated with both thrombin and LPS exhibited a synergistic 3.09 ± 0.71 fold increase in IL-8 secretion compared to LPS alone, and a synergistic 4.44 ± 0.50 fold increase in IL-8 secretion compared to thrombin alone (Figure 1). Given the pronounced IL-8 secretion by

HEECs treated for 48hr with thrombin or LPS, either alone or in combination, all subsequent cytokine/chemokine data were collected at this time point.

Next, we sought to delineate HEEC secretion of other cytokines, chemokines and growth factors in response to the thrombin and LPS treatments. As shown in Figure 2, thrombin alone significantly increased HEEC secretion of inflammatory IL-6, G-CSF, MCP-1, GRO-α and IP-10, and significantly reduced secretion of IL-17, when compared to the NT control. Thrombin alone had no significant effect on the remaining factors: TNFα, VEGF, IP-10, IFNγ, GM-CSF, RANTES, IL-1β, IL-10 and MIP-1β (Figure 2). LPS alone significantly increased HEEC secretion of inflammatory IL-6, G-CSF, MCP-1, GRO-α, IP-10, and IFNγ when compared to the NT control, without altering the secretion of TNFα, VEGF, IL-17, GM-CSF, RANTES, IL-1β, IL-10 or MIP-1β (Figure 2). When compared to LPS alone, thrombin significantly and synergistically augmented the LPS-induced HEEC secretion of inflammatory IL-6 (3.14 ± 0.36 fold); G-CSF (12.28 ± 2.19 fold); and GRO-α (1.69 ± 0.14 fold) (Figure 2). When compared to LPS alone, the combination of thrombin and LPS also significantly augmented HEEC secretion of inflammatory MCP-1 (1.75 ± 0.12 fold); TNF-α (1.51 ± 0.09 fold); and VEGF (1.08 ± 0.02 fold), although these were additive responses (Figure 2). Compared to LPS alone combination thrombin and LPS had no significant effect on HEEC secretion of any of the other factors tested. However, compared to thrombin alone, thrombin and LPS significantly augmented HEEC secretion of IL-17 (1.38 ± 0.07 fold); IP-10 (1.36 ± 0.07 fold); IFNγ (1.35 ± 0.08 fold); and GM-CSF (1.07 ± 0.02 fold) (Figure 2). IL-12 and MIP-1α were undetectable for all treatments (data not shown).

Thrombin synergistically augments LPS-induced HEEC inflammation through PAR-1 activation

Having demonstrated that HEECs treated with thrombin or LPS generate specific inflammatory cytokine/chemokine profiles, and that thrombin augments the HEEC inflammatory response to LPS both synergistically and additively, we sought to clarify the mechanisms involved. Given that thrombin activates PAR1, PAR3, and PAR4 [13], we investigated whether the effects of thrombin on basal and LPS-induced HEEC inflammation was mediated in a PAR-dependent mechanism. As shown in Figure 3A, all four PARs are expressed by HEECs under basal conditions, with PAR1 being the most highly expressed (Figure 3A). Having confirmed PAR1-4 expression by HEECs, their functional role in modulating HEEC responses to LPS was tested using specific agonists. Since a specific PAR3 agonist was not available for these studies, we focused on PAR1 and PAR4. Since thrombin does not activate PAR2 [13], a specific agonist for this receptor was also tested to serve as a negative control. As shown in Figure 3(B–D), none of the PAR agonists alone altered HEEC IL-8 secretion. However, the PAR1 agonist significantly and synergistically augmented the LPS-induced IL-8 response by 1.49 ± 0.01 fold when compared to LPS alone (Figure 3B). In contrast, neither the PAR2 (Figure 3C) nor the PAR4 (Figure D) agonist significantly altered the HEEC IL-8 response to LPS.

Since the PAR1 agonist augmented LPS-induced HEEC IL-8, similarly to thrombin, the ability of this agonist to modulate other HEEC cytokine/chemokine responses to LPS was examined. In contrast to thrombin, the PAR1 agonist alone had no effect on the basal

secretion of any of the factors tested; consequently no additive effects of the PAR1 agonist and LPS was observed as we had with thrombin and LPS on factors such as MCP-1, TNFα, and VEGF (Figure 4). However, similarly to thrombin, pretreatment of HEECs with the PAR1 agonist significantly and synergistically augmented LPS-induced secretion of IL-6 (2.54 ± 0.27 fold); G-CSF (2.48 ± 0.21 fold); and GRO-α (1.23 ± 0.03 fold) when compared to LPS alone (Figure 4).

Thrombin modulates HEEC suppressors of cytokine signaling expression

Having found that thrombin, through PAR1 activation, acts to synergistically augment the secretion of specific HEEC cytokines and chemokines in response to LPS, we further investigated the mechanisms involved. Since LPS responses are mediated by TLR4, we first sought to determine if thrombin increased HEEC TLR4 expression. As shown in Figure 5A, treatment of HEECs with either thrombin or LPS, alone or in combination had no effect on TLR4 mRNA levels. We next examined whether thrombin altered the expression of TLR inhibitors to allow TLR4 activation to cause a more aggravated inflammatory response. Since the suppressors of cytokine signaling 1 (SOCS1) and SOCS3 proteins inhibit TLR4-mediated induction of inflammatory factors [25], their expression in HEECs in response to thrombin and the PAR1 agonist was investigated. As shown in Figure 5B treatment of HEECs with thrombin or the PAR1 agonist significantly increased SOCS1 protein expression when compared to the NT control (Figure 5B). Treatment of HEECs with thrombin significantly decreased SOCS3 protein expression by 26.9 ± 8.3% compared to the NT control, however the PAR1 agonist had no effect on SOCS3 protein expression (Figure 5B).

Discussion

Bacterial infection and placental abruption are important risk factors for preterm birth [9]. Therefore, the objective of this study was to provide further insight into the relationship between coagulation and inflammation in the uterine endothelium. Bacterial infections gain access to the maternal-fetal interface either by ascending into the uterus from the lower reproductive tract; by disseminating via the maternal circulation from a distant site; or rarely, by descending into the uterus from the peritoneal cavity [26]. The endothelium is the first tissue exposed to invasive pathogens in the bloodstream. Its response to infection or tissue injury is to become activated to recruit immune cells and express adhesion molecules that allow their extravasation into tissues for host defense [15]. Thus, this study questioned the impact a systemic bacterial infection would have on the uterine endothelium in the context of excess thrombin production arising from abruption.

We previously demonstrated that bacterial LPS isolated from *Escherichia coli* 055:B5 induced an inflammatory response in HEECs by upregulating their secretion of the cytokine IL-6; the chemokine IL-8; and the growth factor G-CSF, via TLR4 activation [17]. In this current study we examined the HEEC inflammatory response to bacterial LPS isolated from *Escherichia coli* 0111:B4 at the same dose [17]; thrombin; or both in combination. The bacterial LPS used in this study not only upregulated the secretion of IL-6, IL-8 and G-CSF as previously reported [17], but also increased secretion of MCP-1, GRO-α, IP-10 and IFNγ,

although the IP-10 and IFNγ responses were very mild. The production of these additional inflammatory mediators is likely due to a difference in the strain of bacterial LPS used [27]. Thrombin alone induced a similar, yet distinct, inflammatory profile in the HEECs by upregulating their secretion of IL-6, IL-8, G-CSF, MCP-1, GRO-α and IP-10 while reducing basal levels of IL-17. Furthermore, most of these responses were less robust than those induced by LPS. This suggests that LPS and thrombin induce HEEC inflammation through distinct mechanisms. Our findings are, however, in keeping with studies in endothelial cells from other sites, such as the brain and umbilical cord, showing that thrombin induces a similar chemokine profile to the HEECs [28–30].

When HEECs were pretreated with thrombin and then exposed to LPS, two primary responses were observed: a synergistic upregulation of IL-6, IL-8, G-CSF and GRO-α secretion; and an additive upregulation of MCP-1, TNFα and VEGF secretion. Interestingly, there were no changes in the levels of IL-1β secretion in HEECs treated with LPS or thrombin, either alone or in combination. This was surprising since IL-1β is an important mediator of preterm birth [31–33], and thrombin induces IL-1β in microglia and astrocytes [34, 35]. However, to our knowledge, no other studies have reported thrombin-induced IL-1β production by the endothelium [30]. Instead, our results suggest that under conditions of infection and/or abruption, HEECs produce factors such as IL-8, MCP-1, GRO-α and G-CSF that are important for the recruitment and survival of immune cells such as monocytes [36]. Once they undergo diapedesis and entered the decidual tissue, it is these peripheral-derived macrophages and other immune cells [37, 38] that may serve as a localized source of IL-1β [39] in the setting of intrauterine infection and/or intrauterine hemorrhage.

PAR1, PAR3 and PAR4 are all stimulated by thrombin [13]. HEECs express all four receptors, with PAR1 being predominant, which in keeping with their expression in other endothelial cells [40]. The synergistic effect of thrombin on HEEC inflammatory responses to LPS were mediated by PAR1, since a specific receptor agonist induced the same synergistic augmentation of these factors by HEECs when exposed to LPS. However, the additive effect of thrombin on HEEC responses to LPS was independent of PAR1 since the receptor agonist alone had no effect on HEEC basal cytokine/chemokine production, unlike thrombin alone. Thus, another pathway may also be involved that mediates the effects of thrombin alone on HEEC cells and is responsible for the additive inflammatory response seen in combination with LPS. Thus, it remains possible that PAR3, together with PAR1, mediates the additional modulation of the LPS-induced inflammation. Alternatively, studies in amniotic mesenchymal cells showed that thrombin induced cell activation directly via PAR1 and potentially indirectly via TLR4 [41].

How thrombin exerts its synergistic effects on HEEC responses to LPS downstream of PAR1 activation was also examined. PAR1 stimulation can activate the NFκB pathway [42], and we speculated that this might lead to augmented TLR4 function by modulating receptor expression. However, our findings suggest that TLR4 expression was not altered in HEECs by the presence of thrombin or LPS, either alone or in combination, and thus was not the cause for the augmented inflammatory response. Instead we found that the negative regulator of TLR4 signaling, SOCS1 and SOCS3 [25] were modulated. At the protein level, while thrombin downregulated HEEC SOCS3 expression, this was not altered in response to

PAR1 activation by its agonist. Thus, while suppression of SOCS3 may play a role in the HEEC LPS-mediated inflammation, it may not be the mechanism by which PAR1 augments this response. Of interest other studies investigating the role of thrombin and SOCS3, found that thrombin and PAR1 activation directly stimulated SOCS3 expression in microglia and suppressed the LPS response in microglia [43, 44].

We acknowledge that the maternal-fetal interface involves the placental trophoblast, the decidual stroma, and the uterine endothelium, and each of these components may individually participate in the pathogenesis of preterm birth. While we recognize the multiple pathways may contribute to the inflammatory cascade in response to thrombin and bacterial LPS, the focus our investigation was at the uterine microvasculature. Another limitation of our study is that our experimental model examined HEECs exposed to thrombin prior to LPS. While our results clearly demonstrate that thrombin actively alters the LPS inflammatory response, whether there is a similar effect seen when thrombin and LPS are combined simultaneously, or when HEECs are exposed to LPS before thrombin would warrant further study.

In summary, this study demonstrates that thrombin and LPS induce distinct inflammatory profiles in HEECs. Moreover, thrombin, via PAR1 activation, synergistically augments LPS-induced production of chemokines involved in immune cell recruitment and survival. These findings suggest a mechanism by which preterm birth in the context of intrauterine abruption and bacterial infection may be associated with an aggravated uterine inflammatory response.

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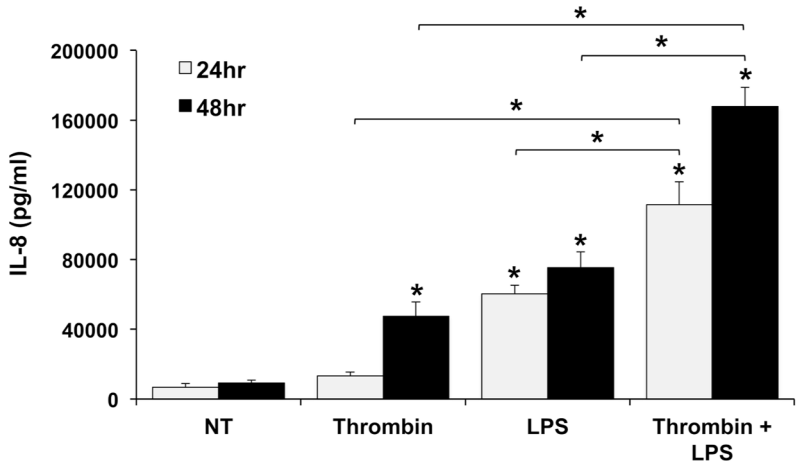

Figure 1. Thrombin augments basal and LPS-induced IL-8 secretion by HEECs in a time-dependent manner

HEECs were pretreated with no treatment (NT) or thrombin (0.25 U/ml) for 1hr followed by NT or treatment with LPS (1μg/ml) for 24 hours (n=4) and 48 hours (n=5). Supernatants were collected and IL-8 measured by ELISA. *p<0.05 vs. the NT control unless indicated otherwise.

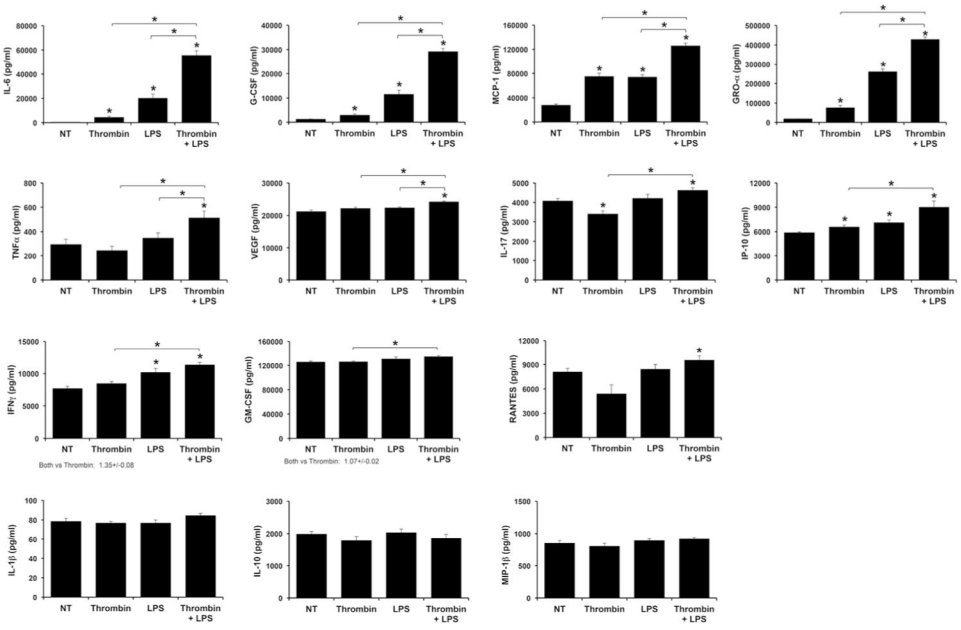

Figure 2. Thrombin augments basal and LPS-induced HEEC cytokine and chemokine secretion by HEECs

HEECs were pretreated with no treatment (NT) or thrombin (0.25 U/ml) for 1hr followed by NT or treatment with LPS (1μg/ml) for 48 hours (n=4). Supernatants were collected and measured by multiplex analysis. Barcharts IL-6, G-CSF, MCP-1, GRO-α, TNFα, VEGF, IL-17, IP-10, IFNγ, GM-CSF, RANTES, IL-1β, IL-10 and MIP-1β secretion. *p<0.05 vs. the NT control unless indicated otherwise.

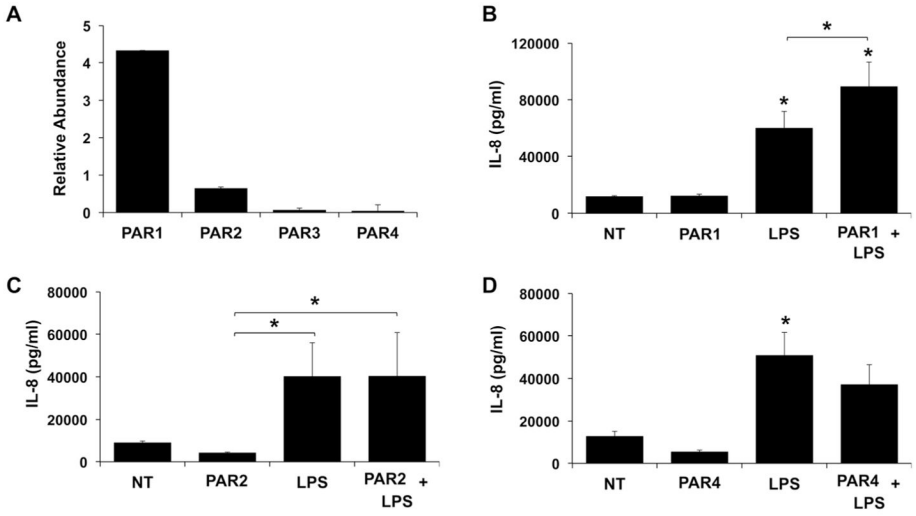

Figure 3. Expression and function of PAR receptors in HEECs

(A) RNA was isolated from untreated HEEC cultures (n=3). qRT-PCR was performed for PAR1-4. PAR mRNA levels are expressed as relative abundance after normalization to GAPDH. (B–D) HEECs were pretreated for 1hr with no treatment (NT) or either: (B) a PAR-1 agonist (1mM); (C) a PAR-2 agonist (1mM); or (D) a PAR-4 agonist (1mM). All cells were then treated with either NT or LPS (1μg/ml) for 48 hours (n=3). Supernatants were collected and IL-8 measured by ELISA. *p<0.05 vs. the NT control unless indicated otherwise.

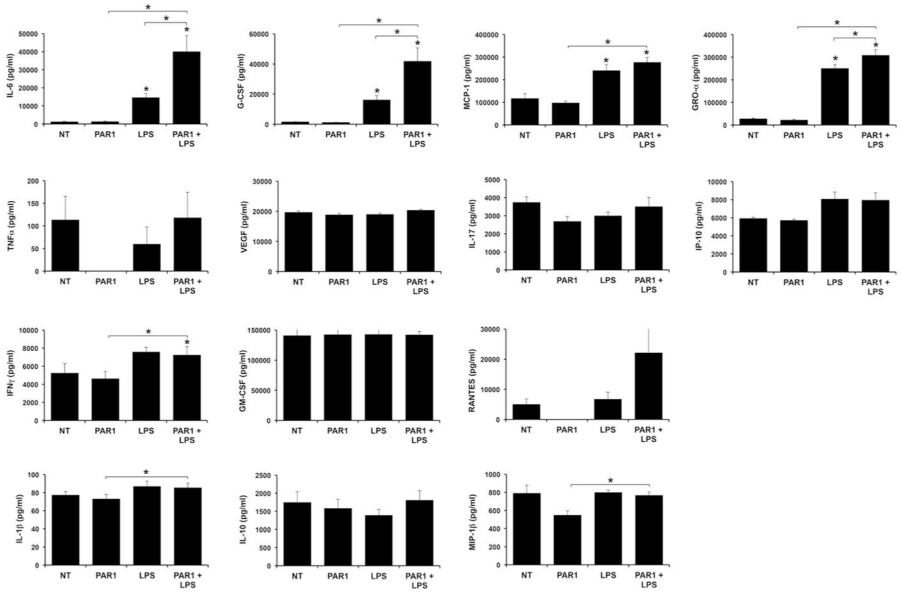

Figure 4. PAR1 agonist augments LPS-induced HEEC cytokine and chemokine secretion by HEECs

HEECs were pretreated with no treatment (NT) or a PAR-1 agonist (1mM) for 1hr followed by NT or treatment with LPS (1μg/ml) for 48 hours (n=3). Supernatants were collected and measured by multiplex analysis. Barcharts IL-6, G-CSF, MCP-1, GRO-α, TNFα, VEGF, IL-17, IP-10, IFNγ, GM-CSF, RANTES, IL-1β, IL-10 and MIP-1β secretion. IL-12 and MIP-1α were below the assay's detection limit. *p<0.05 vs. the NT control unless indicated otherwise.

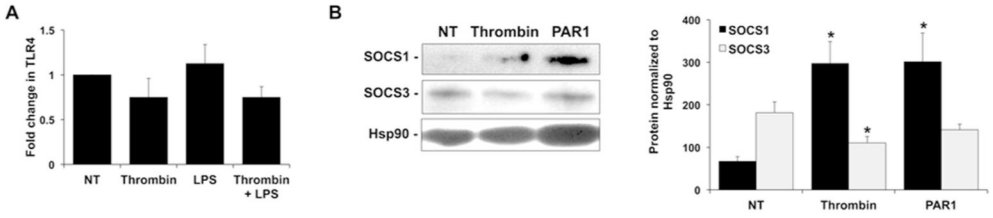

Figure 5. Effect of thrombin on HEEC TLR4 and SOCS expression

(A) HEECs were pretreated with no treatment (NT) or thrombin (0.25 U/ml) for 1hr followed by NT or treatment with LPS (1μg/ml) for 24 hours (n=3). Cellular RNA was isolated and qRT-PCR performed for TLR4. Barchart shows TLR4 mRNA expressed as fold change relative to the NT control. (B) HEECs were treated with either no treatment (NT), thrombin (0.25 U/ml) or a PAR-1 agonist (1mM) for 24 hours after which protein was isolated and Western blot performed for SOCS1, SOCS3 and Hsp90. Blots are from one representative experiment. Barchart shows SOCS1 and SOCS3 protein expression determined by densitometry and normalized to Hsp90 (*p<0.05 vs. NT; n=3–5).