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Thrombin inhibits HMGB1-mediated proinflammatory signaling responses when endothelial protein C receptor is occupied by its natural ligand

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High mobility group box 1 (HMGB1) is involved in the pathogenesis of vascular diseases. Unlike activated protein C (APC), the activation of PAR-1 by thrombin is known to elicit proinflammatory responses. To determine whether the occupancy of EPCR by the Gla-domain of APC is responsible for the PAR-1-dependent antiinflammatory activity of the protease, we pretreated HUVECs with the PC zymogen and then activated PAR-1 with thrombin. It was found that thrombin down-regulates the HMGB1-mediated induction of both TNF-α and IL-6 and inhibits the activation of both p38 MAPK and NF-κB in HUVECs pretreated with PC. Furthermore, thrombin inhibited HMGB1-mediated hyperpermeability and leukocyte adhesion/migration by inhibiting the expression of cell adhesion molecules in HUVECs if EPCR was occupied. Collectively, these results suggest the concept that thrombin can initiate proinflammatory responses in vascular endothelial cells through the activation of PAR-1 may not hold true for normal vessels expressing EPCR under *in vivo* conditions. [BMB Reports 2013; 46(11): 544-549]

INTRODUCTION

High-mobility group box 1 protein (HMGB1) is a promising therapeutic target for the treatment of severe vascular inflammatory diseases, such as sepsis and septic shock (1, 2). In fact, the blockade of HMGB1, even at later times after the onset of infection, has been shown to rescue mice from lethal sepsis (3, 4). HMGB1 has both intra- and extracellular pleiotropic effects. In the cell nuclei, HMGB1 binds DNA, and promotes protein assembly on DNA targets (1, 5), and when released from necrotic or activated cells, extracellular HMGB1 triggers inflammation, immune responses, and tissue regeneration (1, 2, 5). HMGB1 also acts as a lethal mediator in conditions such as sepsis, in which serum HMGB1 levels are substantially increased (2, 4). Once released into the extracellular milieu, HMGB1 can bind to cell surface receptors, such as toll-like receptors (TLR) 2 and 4, and the receptor for advanced glycation end products (RAGE) (2, 4) to transduce intracellular signals and to elicit cellular responses, including chemotaxis and pro-inflammatory cytokine release (6, 7).

Thrombin, a plasma serine protease, plays a central role in the formation of blood clots by cleaving fibrinogen to fibrin (8), and has also diverse biological regulatory activities related to inflammation, allergy, tumor growth, metastasis, apoptosis, tissue remodeling, and thrombosis (9-12). Thrombin can initiate its cellular effects primarily through the activation of protease-activated receptors (PAR) 1 and 4 expressed on the surface of various cell types (11, 13). The extracellular domain of PAR-1 contains a high affinity, hirudin-like binding site for thrombin on the C-terminal end of the scissile bond, thus it is recognized and rapidly cleaved by thrombin at a much higher specificity than PAR-4 (9). It is generally thought that the activation of PAR-1 by thrombin initiates proinflammatory signaling responses on vascular endothelial cells (11, 13-15). In contrast to thrombin, the activation of PAR-1 by activated protein C (APC) elicits anti-inflammatory responses on vascular endothelial cells (16). In this case, however, the protective effect of APC requires the γ-carboxyglutamic acid (Gla) domain-dependent interaction of the protease with endothelial protein C receptor (EPCR) (17). In a recent study, we discovered that both EPCR and PAR-1 are associated with caveolin-1 within the lipid rafts of human umbilical vein endothelial cells (HUVECs) (18) and that the occupancy of EPCR by either APC or the zymogen protein C leads to dissociation of EPCR from caveolin-1 and the recruitment of PAR-1 to a protective signaling pathway independent of the protease activating PAR-1 (19). Thus, the activation of PAR-1 by thrombin initiated protective signaling responses in HUVECs stimulated by proinflammatory cytokines (19). In light of our recent findings that APC potently inhibits HMGB1 secretion and HMBG1-mediated proinflammatory signaling responses in cytokine-stimulated HUVECs, in this study, we decided to examine the effect of

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thrombin on HMGB1-mediated inflammatory responses in human endothelial cells under conditions in which the cell surface receptor, EPCR, is occupied with its natural ligand, protein C. Our results demonstrate that the state of EPCR occupancy determines the signaling specificity of the PAR-1 cleavage. Thus, when EPCR is ligated by protein C, the activation of PAR-1 by thrombin inhibits all HMGB1-mediated proinflammatory signaling responses in HUVECs.

RESULTS AND DISCUSSION

Effects of thrombin + PC-S195A on LPS-induced HMGB1 release

Previous studies have demonstrated that LPS stimulates HMGB1 release from murine macrophages and human endothelial cells (20-22). Chen *et al*. (23) showed that an LPS concentration of 100 ng/ml optimally induces HMGB1 release by macrophages. Similarly, in this study we found that the same concentration of LPS is sufficient to optimally stimulate HMGB1 release by HUVECs (Fig. 1A). It is generally thought that thrombin induces proinflammatory responses by cleaving PAR-1 at the surface of endothelial cells (11, 13-15). On the other hand, the cleavage of the same receptor by APC is known to elicit antiinflammatory responses (16). It has been demonstrated that the PAR-1-dependent antiinflammatory activity of APC requires the protease to form a complex with EPCR on endothelial cells (16). Both APC and the protein C zymogen bind to EPCR via their Gla-domains and the affinity of both molecules for the receptor is essentially identical (17). In a series of recent studies, we have demonstrated that the occupancy of EPCR by its natural ligand protein C/APC can switch the PAR-1-dependent signaling specificity of thrombin from a proinflammatory to an antiinflammatory response in HUVECs (19, 24-27). Therefore, we decided to investigate the effect of thrombin on the LPS-induced HMGB1 release by HUVECs under experimental conditions in which EPCR is occupied by its natural ligand protein C. To simplify the interpretation of results, we pretreated cells with the catalytically inactive PC-S195A (50 nM for 30 min) which binds to EPCR normally but even if activated to APC-S195A, the mutant is incapable of activating PAR-1 (24). Thus, the PC-S195A-pretreated cells were incubated with increasing concentrations of thrombin (0.1-10 nM for 3 h) followed by their stimulation with 100 ng/ml LPS for 16 h. The results presented in Fig. 1A show that pretreatment of endothelial cells with PC-S195A results in thrombin inhibiting the proinflammatory effect of HMGB1 with its maximal effect peaking at thrombin concentrations of 1-2 nM. Thrombin alone did not affect HMGB1 release (Fig. 1A). As presented in Fig. 1B, the inhibitory effect of thrombin + PC-S195A on HMGB1 release required both EPCR and PAR-1, since both cleavage-blocking PAR-1 and function-blocking EPCR antibodies (Fig. 1B) but not corresponding control antibodies (data not shown) abrogated HMGB1 release.

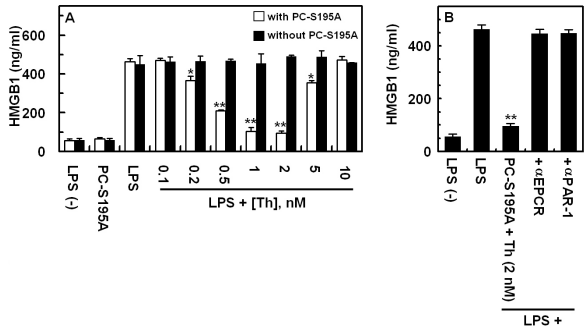

Fig. 1. Effect of thrombin on LPS-mediated HMGB1 release by HUVECs. (A) HUVECs were pretreated with (open bar) or without (closed bar) PC-S195A (50 nM for 30 min) followed by incubation with indicated concentrations of thrombin (3 h) and stimulation with LPS (100 ng/ml for 16 h). HMGB1 release was measured by an ELISA as described under "Materials and Methods". (B) The same as (A) except that cells were preincubated with function-blocking anti-EPCR (αEPCR) or anti-PAR-1 (αPAR-1) antibodies for 30 min. Results are expressed as means ± S.D. of at least three experiments. *P < 0.05 or **P < 0.01 vs. LPS alone.

High plasma concentrations of HMGB1 in patients with inflammatory diseases, such as, sepsis or septic shock, correlate with poor prognosis and high mortality (28). Furthermore, the pharmacological inhibition of HMGB1 is known to improve survival in animal models of acute inflammation induced in response to an endotoxin challenge (29). Therefore, the prevention of the LPS-induced HMGB1 release by thrombin + PC-S195A may suggest that low concentrations of thrombin, sufficient to activate PAR-1, can exert a protective effect in intact vasculature expressing EPCR. However, this would not be the case if the vessel wall lacks EPCR (due to either damage or down-regulation by cytokines) in which case the activation of PAR-1 by thrombin would elicit proinflammatory responses as has been reported.

Effect of thrombin + PC-S195A on HMGB1-mediated permeability

A previously described permeability assay was used to examine the effect of thrombin + PC-S195A on HUVECs barrier integrity (19). HMGB1 is known to disrupt barrier integrity in endothelial cells (30, 31). To evaluate the effect of thrombin on HMGB1-mediated barrier permeability, HUVECs were treated with various concentrations of thrombin (0.1-10 nM) in the presence of PC-S195A (50 nM) before stimulating cells with HMGB1 (1 μg/ml, 16 h). As shown in Fig. 2A, thrombin + PC-S195A exhibited a barrier protective effect, thus dose-dependently decreasing HMGB1-mediated enhancement of permeability in endothelial cells. And the inhibitory effect of thrombin + PC-S195A on HMGB1-mediated permeability required both EPCR and PAR-1, since function-blocking antibodies against both receptors abrogated HMGB1-mediated enhancement in permeability (Fig. 2B).

HMGB1 induces barrier disruptive responses by promoting the phosphorylation of p38 MAPK (32, 33). To determine whether the protective effect of thrombin + PC-S195A is mediated

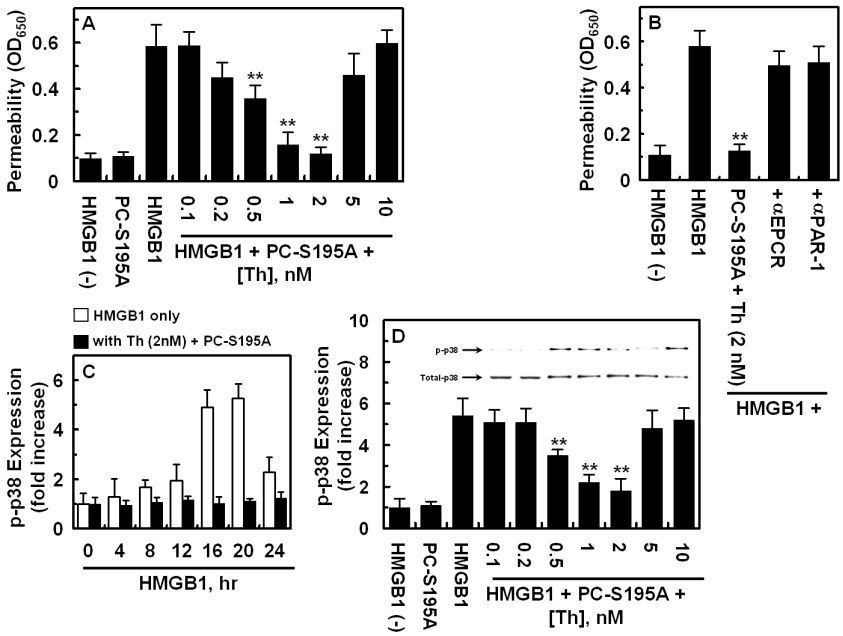

Fig. 2. Effect of thrombin on HMGB1-mediated barrier permeability when EPCR is occupied. (A) The endothelial cell permeability in response to HMGB1 (1 μg/ml for 16 h) was monitored as a function of different concentrations of thrombin (3 h) with or without preincubation of cell monolayers with PC-S195A (50 nM for 30 min). The barrier permeability was monitored by measuring the flux of Evans blue bound albumin across HUVECs as described under "Materials and Methods". (B) The same as (A) except that cells were preincubated with function-blocking anti-EPCR (αEPCR) or anti-PAR-1 (αPAR-1) antibodies for 30 min. (C) HUVECs were preincubated with or without thrombin (2 nM for 3 h) and PC-S195A (50 nM for 30 min) followed by incubation with HMGB1 (1 μg/ml) for indicated periods. Then, phosphor-p38 expression was measured by an ELISA. (D) HUVECs were treated as described in (A) and HMGB1-mediated phosphor-p38 expression was measured by an ELISA. Insert image; western blotting for p-p38 and total p38 (from left lane; HMGB1 (-), PC-S195A (50 nM), HMGB1 (1 μg/ml), HMGB1 + PC-S195A + Th (0.2 nM), HMGB1 + PC-S195A + Th (0.5 nM), HMGB1 + PC-S195A + Th (2 nM), HMGB1 + PC-S195A + Th (5 nM). Results are expressed as means ± S.D. of at least three experiments. **P < 0.01 vs. HMGB1 alone.

through the inhibition of the HMGB1-mediated activation of the p38 MAPK pathway, we preincubated HUVECs with thrombin + PC-S195A before stimulation with HMGB1 and then examined phospho-p38 MAPK levels by an ELISA and western blotting analysis. As shown in Fig. 2C, D, HMGB1 up-regulated the expression of phosphorylated p38 transiently between 16 and 20 h, and thrombin + PC-S195A significantly inhibited this up-regulation. These findings show that the occupancy of EPCR by protein C changes the PAR-1-dependent signaling specificity of thrombin by inhibiting the HMGB1-mediated activation of the proinflammatory p38 MAPK pathway.

Effects of thrombin + PC-S195A on HMGB1-mediated CAM expression, THP-1 cell adhesion, and migration

Previous studies have shown that HMGB1 mediates inflammatory responses by increasing the cell surface expression of CAMs, such as ICAM-1, VCAM-1, and E-selectin on the surface of endothelial cells, thereby promoting the adhesion and migration of leukocytes across the endothelium and eventually to sub-endothelial inflammatory sites (34-37). In the present study, we found that HMGB1 up-regulates the expression of VCAM-1, ICAM-1, and E-Selectin at both protein (Fig. 3A), and transcription (mRNA expression, Fig. 3B) levels and that thrombin inhibits the induction of all three CAMs by a concentration-dependent manner if HUVECs were pretreated with PC-S195A. It has been known that elevated CAM expression is associated with enhanced leukocyte binding and migration. To further demonstrate that the occupancy of EPCR by its natural ligand protein C switches the signaling specificity of thrombin from a proinflammatory to an antiinflammatory response, we monitored the adhesion and migration of the monocytic THP-1 cell line across the HMGB1-activated HUVEC monolayers. Thrombin + PC-S195A down-regulated the THP-1 cell adherence and their subsequent migration across the HMGB1- stimulated endothelial cells in a concentration-dependent manner (Fig. 3C and D). Furthermore, these protective effects were PAR-1 and EPCR dependent since function-blocking antibodies to both receptors abrogated the protective effect of thrombin in PC-S195A pretreated cells (data not shown). These results suggest that thrombin in the presence of PC-S195A not only inhibits the endotoxin-mediated release of HMGB1 by endothelial cells, but also down-regulates the proinflammatory signaling effect of the released HMGB1, thus inhibiting the amplification of inflammatory pathways by the nuclear cytokine.

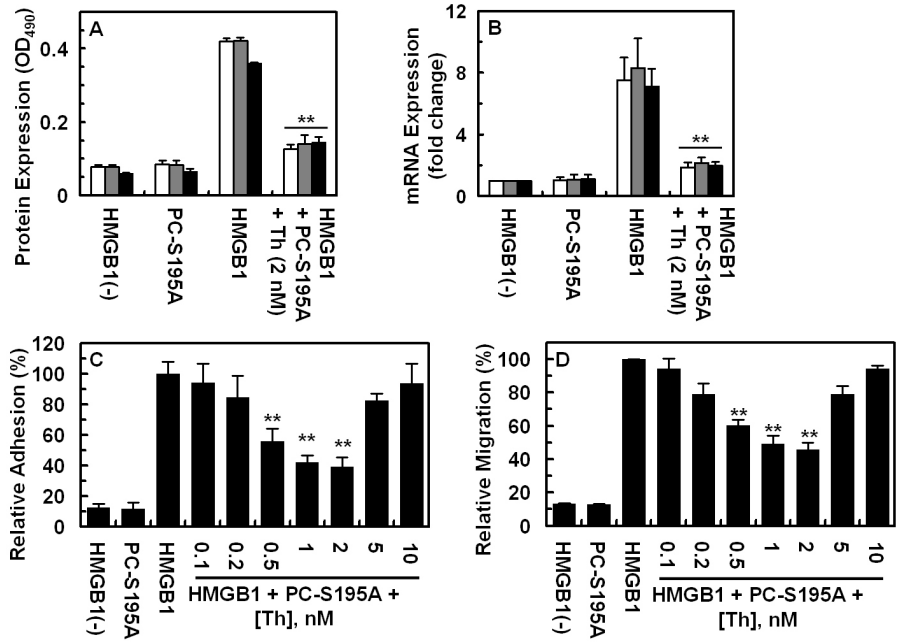

Fig. 3. Effect of thrombin + PC-S195A on HMGB1-mediated expression of adhesion molecules. HMGB1-mediated (1 μg/ml) expressions of VCAM-1 (white bar), ICAM-1 (gray bar), and E-selectin (black bar) at protein (ELISA, A) and transcription (mRNA; RT-PCR, B) levels in HUVECs were analyzed after pretreating cells with PC-S195A (50 nM for 30 min) and incubating them with thrombin (2 nM) for 3 h. (C) HMGB1 (1 μg/ml for 16 h)-mediated adherence of monocytes to HUVEC monolayers was analyzed after pretreating cells with PC-S195A (50 nM for 30 min) and activating them with different concentrations of thrombin for 3 h. (D) HUVECs were treated as in (C) and the HMGB1-mediated migration of monocytes through HUVEC monolayers was analyzed as described under "Materials and Methods". Results are expressed as means ± S.D. of at least three experiments. **P < 0.01 vs. HMGB1 alone.

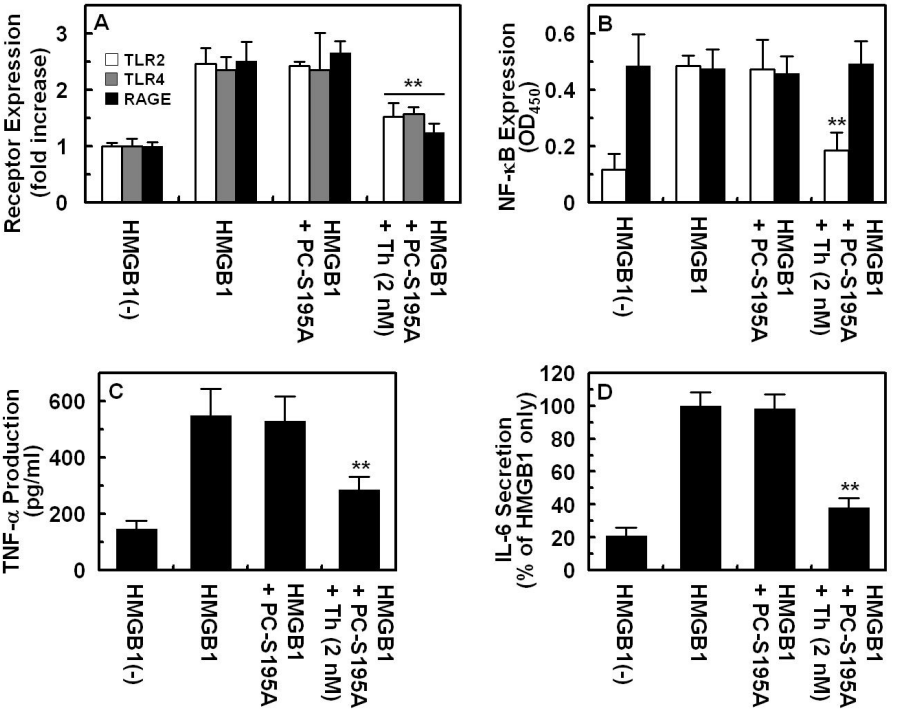

Fig. 4. Effects of thrombin + PC-S195A on HMGB1-mediated expression of proinflammatory receptors and cytokines. (A) HUVECs were stimulated with HMGB1 with or without pretreatment with either PC-S195A alone (50 nM for 30 min) or PC-S195A + thrombin (2 nM) followed by analysis of the expression of HMGB1 receptors (TLR2, TLR4 and RAGE) by an ELISA as described under "Materials and Methods". (B) HUVECs were treated as in A followed by monitoring the activity of total NF-κB (black bar) and activated NF-κB p65 (open bar). (C and D) The same as above except that the expression of TNF-α (C) and IL-6 (D) were analyzed by ELISA-based assays. Results are expressed as means ± S.D. of at least three experiments. **P < 0.01 vs. HMGB1 alone.

Effect of thrombin + PC-S195A on the expression of HMGB1 receptors, NF-κB activation, and HMGB1-mediated induction of IL-6 and TNF-α

We next investigated the effect of HMGB1 on the expression of its own receptors and the effect of thrombin + PC-S195A on the expression of these receptors by endothelial cells. As presented in Fig. 4A, HMGB1 induced the expression of TLR2, TLR4, and RAGE on endothelial cells by 2.5 fold. However, thrombin +

PC-S195A significantly inhibited the stimulatory effect of HMGB1 on all three receptors (Fig. 4A). Like HMGB1, LPS also stimulated the expression of TLR2, TLR4, and RAGE on the surface of endothelial cells, but thrombin in the presence of PC-S195A inhibited the LPS-mediated expression of all three receptors (data not shown).

NF-κB activation is known to be responsible for the initiation of proinflammatory responses and the induction of cytokines such as TNF-α and IL-6 by endothelial cells (38-41). As presented in Fig. 4B, we found that HMGB1 effectively enhances the activity of NF-kB (Fig. 4B) and the production of TNF-α and IL-6 in endothelial cells (Fig. 4C and D). In agreement with results presented above, we found that thrombin significantly decreased TNF-α and IL-6 levels and NF-κB activation by HMGB1 in endothelial cells if the cells were pretreated with PC-S195A (Fig. 4 B-D). These results support the conclusion that the occupancy of EPCR by its natural ligand, protein C, shuts down all PAR-1-dependent proinflammatory effects which have been attributed for thrombin in *in vitro* cell culture systems.

Based on results presented in this manuscript, we hypothesize that the cleavage of PAR-1 by thrombin on intact endothelium expressing EPCR would only elicit protective signaling responses in the presence of a physiological concentration of the zymogen protein C (70-80 nM). Thus, the in vitro data assuming a PAR-1-dependent proinflammatory effect for thrombin in cellular models may have no physiological relevance. It should, however, be noted that such a PAR-1 and EPCR dependent protective activity for thrombin may be lost if an injury and/or inflammation has led to denudation of the endothelium and/or down-regulation of the cell surface EPCR, in which case thrombin can initiate proinflammatory responses through activation of PAR-1. The hypothesis that a low concentration of thrombin can play a protective role under normal physiological conditions is consistent with the observation that the infusion of a low concentration of thrombin to dogs has conferred protection in response to a lethal dose of endotoxin (42). Thus, based on our results, we hypothesize that the signaling specificity of vascular PAR-1 cleavage is not determined by the protease that cleaves PAR-1 but rather whether or not the vascular cell surface receptor, EPCR, is occupied by its natural ligand protein C/APC.

MATERIALS AND METHODS

See supplementary document for materials and methods.

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