Published in final edited form as:

J Cell Physiol. 2009 June ; 219(3): 744–751. doi:10.1002/jcp.21718.

Concentration dependent dual effect of thrombin in endothelial cells via PAR-1 and PI3 kinase

Jong-Sup Bae^{1,*}, Yong-ung Kim¹, Moon-Ki Park¹, and Alireza R. Rezaie²

¹Department of Herbal Pharmaceutical Engineering, College of Herbal Bio-Industry, Daegu Haany University, Gyeongsan 712–715 Republic of Korea

²Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, Missouri 63104 USA

Abstract

Disruption of endothelial barrier is a critical pathophysiological factor in inflammation. Thrombin exerts a variety of cellular effects including inflammation and apoptosis through activation of the protease activated receptors (PARs). The activation of PAR-1 by thrombin is known to have a bimodal effect in endothelial cell permeability with a low concentration (pM levels) eliciting a barrier protective and a high concentration (nM levels) eliciting a barrier disruptive response. It is not known whether this PAR-1-dependent activity of thrombin is a unique phenomenon specific for the in vitro assay or it is part of a general anti-inflammatory effect of low concentrations of thrombin that may have a physiological relevance. Here, we report that low concentrations of thrombin or of PAR-1 agonist peptide induced significant anti-inflammatory activities. However, relatively high concentration of thrombin or of PAR-1 agonist peptide showed pro-inflammatory activities. By using function-blocking anti-PAR-1 antibodies and PI3 kinase inhibitor, we show that the direct anti-inflammatory effects of low concentrations of thrombin are dependent on the activation of PAR-1 and PI3 kinase. These results suggest a role for cross communication between PAR-1 activation and PI3 kinase pathway in mediating the cytoprotective effects of low concentrations of thrombin in the cytokine-stimulated endothelial cells.

Keywords

Thrombin; Endothelium; inflammation; PAR-1; PI3 kinase

Introduction

Thrombin, in addition to playing a central role in the formation of blood clots by cleaving fibrinogen to fibrin, possesses diverse biological regulatory activities related to inflammation, allergy, tumor growth, metastasis, apoptosis, and tissue remodeling (Cirino et al., 2000; Coughlin, 2000; Coughlin, 2001; Grand et al., 1996; Klepfish et al., 1993; Macfarlane et al., 2001; Nierodzik et al., 1992). The role of thrombin in inflammation largely is dependent on its ability to regulate the activities of cells such as leukocytes (Kaplanski et al., 1998), platelets (Nierodzik et al., 1992; Nierodzik et al., 1991), and endothelial cells (Kaplanski et al., 1998; Klepfish et al., 1993). Thrombin mediates most of its cellular effects through activation of a series of G protein-coupled receptors known as protease-activated receptors (PARs) which are

* Corresponding Author: Department of Herbal Pharmaceutical Engineering, College of Herbal Bio-Industry, Daegu Haany University 290 Yugok-dong, Gyeongsangbuk-do, 712–715, Republic of Korea Phone: 82–53–819–1498 Fax: 82–53–819–1272 E-mail: BaeJS@dhu.ac.kr.

expressed on the surface of various cell types (Coughlin, 2005; Ossovskaya et al., 2004). To date, four members of the PAR family (PAR-1, -2, -3 and -4) have been identified with distinct N-terminal exodomains which contain the cleavage site for thrombin (Coughlin, 2005; Ossovskaya et al., 2004; Steinhoff et al., 2005). Whereas PAR-1, PAR-3 and PAR-4 are targets for thrombin, cathepsin G, and trypsin (Ishihara et al., 1997; Kahn et al., 1998; Vu et al., 1991; Xu et al., 1998), PAR-2 is activated by trypsin, tryptase, acrosin and coagulation factors Xa and VIIa but not by thrombin (Camerer et al., 2000; Molino et al., 1997; Nystedt et al., 1995; Smith et al., 2000; Steinhoff et al., 1999). Subsequent to the identification of PAR-1 (Rasmussen et al., 1991; Vu et al., 1991), the multiple cellular effects of thrombin could be attributed to its activation of PAR-1 on different cell types, including its effect on leukocyte trafficking, vasoregulation, platelet aggregation, angiogenesis and barrier integrity of endothelial cell (Chin et al., 2003; Cunningham et al., 2000; Ludwicka-Bradley et al., 2000; Naldini and Carney, 1996; Sambrano et al., 2001; Sugama et al., 1992; Suk and Cha, 1999; Vergnolle et al., 1999). PAR-1 is activated when thrombin binds to the extracellular NH₂-terminal domain to catalyze the cleavage of the receptor between the arginine-41 and serine-42 peptide bond (Ossovskaya et al., 2004; Steinhoff et al., 2005). This enzymatic event unmasks a tethered ligand that interacts within sequences corresponding to extracellular loop 2 (amino acid residues 248–268) of the receptor to initiate cellular events (Ossovskaya et al., 2004; Steinhoff et al., 2005). PAR-1 has been detected in a variety of tissues including monocytes, fibroblasts, endothelium, platelets, dental pulp cells, smooth muscle cells, neurons, and certain tumor cell lines (Arena et al., 1996; Chang et al., 1998; Colotta et al., 1994; Grandaliano et al., 1994; Howells et al., 1993; Howells et al., 1994; Vu et al., 1991; Weinstein et al., 1995). In addition, recent observations showed that PAR-1 could regulate vascular function under physiological and pathological conditions (Coughlin, 2000; Coughlin, 2001). In normal human arteries, PAR-1 is confined to the endothelium whereas during atherogenesis, its expression is enhanced in regions of inflammation (Nelken et al., 1992).

Recent studies support a role for thrombin in regulation of inflammation through the activation of PAR-1. Thrombin up-regulates the expression of various mediators and proteins in human umbilical vein endothelial cells (HUVECs) including cytokines (IL-1 and IL-8) (Drake et al., 1992; Ueno et al., 1996), growth factors (EGF-like growth factor, EGF or platelet derived growth factor, PDGF) (Garcia et al., 1993; Kayanoki et al., 1999), and cell adhesion molecules such as E-selectin, P-selectin, intracellular adhesion molecule-1 (ICMA-1) and vascular cell adhesion molecule-1 (VCAM-1) (Kaplanski et al., 1998). Thrombin can evoke inflammatory and immunological responses in endothelial cells by promoting the production of mediators associated with cell adhesion and transendothelial migration. Thrombin is also involved in the induction of apoptosis in neurons, tumor cells and astrocytes through activation of caspases (Donovan et al., 1997).

In studying the action of thrombin in primary endothelial cells, we and others have unexpectedly found a barrier protective activity for a low concentration of thrombin (approximately 50 pM) (Bae et al., 2007b; Feistritzer and Riewald, 2005) which is in contrast with a pro-inflammatory role for thrombin at concentrations above 100 pM. It is not known whether this property of thrombin is a unique phenomenon specific for the in vitro permeability assays or if it is part of a general anti-inflammatory effect for lower concentrations of thrombin that may have a physiological relevance. To address this question, we analyzed the cellular effect of a low concentration of thrombin on the primary vein and artery endothelial cells using various assays suitable for reporting the pro- and anti-inflammatory properties of thrombin in in vitro systems. Our results revealed a potent anti-inflammatory effect for thrombin that reached its maximal effect at 50 pM in human umbilical vein endothelial cells (HUVECs) and 75 pM in human pulmonary artery endothelial cells (HPAECs). These anti-inflammatory activities included barrier protective function, inhibition of leukocyte adhesion and transendothelial migration, and inhibition of expression of cell adhesion molecules (CAM)

such as vascular cell adhesion molecule (VCAM), intracellular adhesion molecules (ICAM) and E-selectin by endothelial cells. We further demonstrate that the anti-inflammatory activities of low concentrations of thrombin are mediated through activation of PAR-1 and phosphatidylinositol 3-kinase (PI3K) pathways.

Materials and methods

Reagents

Cleavage blocking and non-blocking monoclonal anti-PAR-1 antibodies were purchased from Santa Cruz Biologics (Santa Cruz, CA) and used at 25 µg/mL. Tumor necrosis factor-α (TNF-α) was purchased from R&D System (Minneapolis, MN) and used at 10 ng/mL. The thrombin, specific cell permeable phosphatidylinositol 3-kinase inhibitor (LY-294002, used at 10 µM) and bacterial lipopolysaccharide (LPS, used at 10 ng/ml) were obtained from Sigma (St. Louis, MO). The PAR-1 specific thrombin receptor agonist peptide (TFLLRN) was purchased from Bachem Bioscience (Torrance, CA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human pulmonary artery endothelial cells (HPAECs) were obtained from Cambrex Bio Science Inc. (Charles City, IA) and maintained as described before (Bae and Rezaie, 2008; Bae et al., 2007b). Both cells were passaged for no more than 6 generations. Freshly isolated neutrophils were kindly provided by Dr. Jeong Hyun Chang (Daugu Haany University, Gyeongsan-si, South Korea).

Permeability assay

Endothelial cell permeability in response to LPS (10 ng/ml for 4 hours) was quantitated by the spectrophotometric measurement of the flux of Evans blue-bound albumin across endothelial cell monolayers using a modified 2-compartment chamber model as described previously (Bae et al., 2007b).

Cell adhesion assay

Freshly isolated neutrophil adhesion to TNF-α-stimulated HUVECs or HPAECs was evaluated as described (Akeson and Woods, 1993; Bae et al., 2007a; Kim et al., 2001). Briefly, after peripheral blood neutrophils were labeled with 5 µM Vybrant DiD (Molecular Probes), they (1.5×10^6 /ml, 200 µl/well) were added to confluent monolayers of endothelial cells in 96-well plates, which were pretreated with indicated concentrations of thrombin and followed by stimulation with TNF-α (10 ng/ml for 4 hours). The fluorescence of labeled cells was measured (total signal) using a fluorescence microplate reader (Molecular Device). After incubation for 60 min at 37 °C, non-adherent cells were removed by washing four times with pre-warmed media and the fluorescent signals of adherent cells were measured by the same methods. The percentage of adherent neutrophils was calculated by the formula: % adherence = (adherent signal/total signal) × 100.

Transendothelial migration assay (TEM)

Migration of neutrophils through endothelial cells was performed in a dual chamber system as described previously (Bae et al., 2007a). Briefly, endothelial cells (6×10^4) were cultured for 3 days to obtain confluent monolayers which were then incubated with TNF-α (10 ng/ml for 4 hours). Freshly isolated neutrophils (1.5×10^6 /0.2 ml) were added to the upper compartment and incubated at 37°C for 2 hours. Non-migrating cells in the upper chamber were removed by washing and migrating neutrophils on the opposite side of the filter were fixed with 8% glutaraldehyde and stained with 0.25% crystal violet (Sigma). Each experiment was repeated

in triplicate wells, and counting was done in nine randomly selected microscopic high power fields.

ELISA of cell adhesion molecules

The expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin on endothelial cells was determined by a whole-cell ELISA as described (Bae et al., 2007a; Che et al., 2002). Briefly, confluent endothelial cells were treated with indicated concentrations of thrombin followed by stimulation with TNF- α for 4 hours. After the medium was removed, cells were fixed by adding 50 μ l of 1% paraformaldehyde. Then, mouse anti-human monoclonal antibodies to each adhesion molecule (VCAM-1, ICAM-1, E-selectin, 1:50) were added and incubated for 1 hour. Following washing of plates, peroxidase-conjugated anti-mouse IgG antibody (Sigma) was added for 1 hour. The plates were then washed and developed using the substrate *o*-phenylenediamine (Sigma) by monitoring the absorbance at 490 nm.

Results

Effect of varying thrombin concentrations on the barrier protective activity

Primary endothelial cells were incubated with various concentrations of thrombin for 3 hours, followed by washing and incubation with LPS for 4 hours to disrupt the barrier integrity. Fig 1A demonstrates that 25 pM of thrombin exhibits a barrier protective activity with 50 pM showing maximal protective activity. Thrombin concentrations below 10 pM or above 100 pM were ineffective in HUVECs. Similarly, incubation of HPAECs for 3 hours with thrombin at concentrations of 50 to 75 pM potentially reduced permeability in response to LPS (Fig 1C). The function-blocking anti-PAR-1 antibody that blocks the cleavage of PAR-1 by thrombin abolished the barrier protective effect of lower concentrations of thrombin in both primary endothelial cells (Fig 1B,D). Therefore, activation of PAR-1 by lower concentrations of thrombin has a barrier protective activity in human primary endothelial cells.

Effect of varying thrombin concentrations on leukocyte adhesion and transendothelial migration (TEM) in the TNF- α -activated endothelial cells

TNF- α treatment of endothelial cells is associated with up-regulation of leukocyte adhesion and TEM and the mechanism for these effects appear to be mediated through the elevated expression of cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin at the surface of endothelial cells (Dejana, 1996; Frenette and Wagner, 1996; Harlan and Winn, 2002; Huang et al., 2006; Sugama et al., 1992). To determine the effect of different concentrations of thrombin on TNF- α mediated neutrophil adherence to endothelial cells and TEM, primary endothelial cells were incubated with various concentrations of thrombin for 6 hours, followed by washing and then incubating them with 10 ng/ml of TNF- α for 4 hours to activate endothelial cells. Results showed that thrombin concentrations of 25–50 pM in HUVEC and 50–75 pM in HPAEC effectively inhibited neutrophil adhesion (Fig 2A,C) and TEM (Fig 3A,C) and these effects were PAR-1-dependent since a function-blocking anti-PAR-1 antibody abrogated the effects on both cell types (Fig. 2B,D; Fig 3B,D).

Specificity of thrombin effect on the expression of cell adhesion molecules (CAM)

To determine whether the TNF- α mediated neutrophil adhesion and TEM correlate with the elevated expression of CAM and that a low concentration of thrombin blocks CAM expression, we investigated the effect of various concentrations of thrombin on the CAM expression in endothelial cells activated by TNF- α . Interestingly, the TNF- α mediated expression of all three adhesion molecules was inhibited by thrombin at 50 pM in HUVEC and at 75 pM in HPAEC (Fig 4A,C). Further studies were conducted to determine whether the thrombin inhibition of

the expression of these adhesion molecules is mediated through the activation of PAR-1 in TNF- α stimulated endothelial cells. The results presented in Fig 4B and D demonstrate that the inhibition of CAM expression by the low concentration of thrombin was inhibited if cells were preincubated with an antibody that blocks the cleavage of PAR-1 by thrombin. These results clearly demonstrate that the cleavage of PAR-1 by thrombin concentrations of not higher than 75 pM elicits anti-inflammatory responses in endothelial cells.

PAR-1 activation with low concentrations of TFLLRN mimics the anti-inflammatory activities of a low concentration of thrombin

It is known that the thrombin receptor agonist peptide TFLLRN specifically activates PAR-1 on endothelial cells (Ossovska et al., 2004; Steinhoff et al., 2005). To determine whether low concentrations of TFLLRN could mimic similar effects of low concentrations of thrombin we investigated the effect of various concentrations of TFLLRN on endothelial cells using the same assay systems described above. In the primary HUVEC, low concentrations of the PAR-1 agonist peptide (from 25 nM to 50 nM) elicited a barrier protective response (Fig 5A) and in the primary HPAEC, 50 to 75 nM of TFLLRN was required to obtain a similar response (Fig 5B). Further studies revealed that the same range of peptide concentrations inhibited neutrophil adhesion, TEM and CAM expression in both HUVEC and HPAEC, clearly suggesting that the activation of PAR-1 by low concentrations of TFLLRN (not higher than 75 nM) elicits anti-inflammatory responses in endothelial cells.

Involvement of PI3 kinase in the anti-inflammatory activities of low concentrations of thrombin

Previous results have demonstrated that the barrier protective activity of activated protein C (APC) is mediated via the PI3 kinase pathway (Finigan et al., 2005). To determine whether PI3 kinase is also involved in the anti-inflammatory activities of low concentrations of thrombin, endothelial cells were preincubated with specific cell permeable phosphatidylinositol 3-kinase inhibitor, LY-294002. The barrier protective function of thrombin (50 pM in HUVECs or 75 pM in HPAECs) was effectively suppressed by LY-294002 (Fig 6A). In addition to barrier protective function, inhibition of neutrophil adhesion to both endothelial cells and transendothelial migration through both endothelial cells by the same thrombin concentrations were also suppressed by LY-294002 (Fig 6B,C). These results may suggest that, similar to APC, the activation of the PI3 kinase pathway by low concentrations of thrombin (not higher than 75 pM) elicits anti-inflammatory responses in endothelial cells.

Discussion

It is known that the activation of PAR-1 by thrombin elicits proinflammatory responses through the activation of the NF- κ B pathway in cultured endothelial cells (Steinhoff et al., 2005). However, in a series of recent studies we discovered that when endothelial protein C receptor (EPCR) on endothelial cells is occupied by its natural ligand protein C, the cleavage of PAR-1 by thrombin elicits a protective response that reverses the barrier permeability enhancing effect of proinflammatory molecules in stimulated endothelial cells (Bae and Rezaie, 2008; Bae et al., 2007b; Bae et al., 2007c). In the same previous studies, we demonstrated that both EPCR and PAR-1 are associated with caveolin-1 within lipid rafts of endothelial cells and that the occupancy of EPCR by protein C leads to its dissociation from caveolin-1 and coupling of PAR-1 to a pertussis toxin sensitive $G_{i/o}$ -protein, thus the thrombin cleavage of the receptor elicits a barrier protective response in the cytokine stimulated endothelial cells (Bae and Rezaie, 2008; Bae et al., 2007b; Bae et al., 2007c). In a recent study, a barrier protective activity for a low concentration of thrombin (40 pM) was also observed in endothelial cells independent of the EPCR occupancy by protein C (Feistritzer and Riewald, 2005). In the present study, we extended this observation and demonstrated that, in addition to a barrier protective effect, low

concentrations of thrombin elicit a variety of PAR-1-dependent anti-inflammatory responses in endothelial cells via an EPCR independent mechanism. This was evidenced by the observation that 25–75 pM thrombin inhibited the adhesion of freshly isolated leukocytes to primary endothelial cells, inhibited transendothelial migration (TEM) by leukocytes, and inhibited the expression of cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin by primary endothelial cells. Consistent with our previous results (Bae and Rezaie, 2008; Bae et al., 2007b; Bae et al., 2007c), the optimal thrombin concentration to observe a protective effect in HPAECs (75 pM) was ~2-fold higher than that observed in HUVECs (25–50 pM) supporting the hypothesis that endothelial cells of arterial beds have been held together more tightly and that they are more restrictive to liquid and albumin flux than those in the venular beds (Komarova et al., 2007; Majno and Palade, 1961).

It has been previously demonstrated that the EPCR-dependent cytoprotective and anti-inflammatory activity of activated protein C is mediated through the activation of PI3 kinase which leads to the phosphorylation of the G_i-protein coupled sphingosine 1-phosphate receptor 1 (S1P1), thereby eliciting a protective response in endothelial cells (Finigan et al., 2005). The observation that the specific PI3 kinase inhibitor, LY-294002, blocked the protective signaling responses by the low concentrations of thrombin in all in vitro assays presented above suggests that the PAR-1-dependent protective activity of thrombin is also mediated through the activation of the same PI3 kinase pathway. The exact mechanism by which a low concentration of thrombin elicits a protective response through the activation of PAR-1 is not understood. One possibility is that there are two populations of high and low affinity PAR-1 at the surface of endothelial cells with the high affinity receptor being coupled to the G_i-protein and thus low concentrations of thrombin preferentially activating this population of the receptor, thereby initiating an anti-inflammatory response. Nevertheless, the observation that the PAR-1 specific agonist peptide (TFLLRN) also elicited protective responses at a low concentration excludes this possibility. Noting that endothelial cells may have different type of membrane lipid rafts, another possibility is that PAR-1 may be localized to different membrane lipid rafts containing different signaling molecules. In this context, we previously demonstrated that cavolin-1 plays a critical role in the specificity of the PAR-1-dependent signaling activity of thrombin. Thus, it is possible that a readily thrombin-accessible population of PAR-1 has been localized to a membrane microenvironment that is devoid of cavolin-1 and that this population of PAR-1 signals through G_i-protein. A third possibility is that the cellular signaling activity of a low concentration of thrombin is mediated through activation of two receptors PAR-1 and PAR-3 on endothelial cells. In support of this hypothesis, in a recent study we noted that the barrier protective signaling activity of thrombin in cells treated with siRNA for PAR-3 was eliminated, though it had no effect on the proinflammatory signaling effect of a higher concentration of thrombin (Bae and Rezaie, 2008). PAR-3 is not thought to be directly involved in signal transduction in endothelial cells since it lacks a cytosolic domain (Steinhoff et al., 2005). However, it has been recently reported that PAR-1 can form heterodimers with PAR-3 and that the dimerization can modulate the G-protein coupling specificity of PAR-1 in endothelial cells (McLaughlin et al., 2007). Thus, it is possible that the protective activity of a low pM concentration of thrombin is mediated through activation of both PAR-1 and PAR-3 colocalized to specific membrane microenvironments in endothelial cells. Further studies will be required to determine if any one of these or other more complex mechanisms account for the anti-inflammatory effects of low pM concentrations of thrombin in endothelial cells.

In summary the results presented in this study suggest that, similar to its dual procoagulant and anticoagulant roles in the clotting pathway, thrombin can also elicit paradoxical intracellular signaling responses in cultured endothelial cells through the activation of PAR-1. It appears that at concentrations below 100 pM the PAR-1-dependent signaling activity of thrombin is protective and mediated through a PI3 kinase dependent activation of G_i-protein coupled receptors. On the other hand, at concentrations of higher than 100 pM thrombin produces a

pro-inflammatory response through the activation of PAR-1 apparently coupled to G_q and/or G_{12/13} subfamily of G-proteins in endothelial cells. Further studies will be required to understand the exact mechanism by which the protease concentration determines the cellular signaling specificity of thrombin in endothelial cells.

Acknowledgements

We would like to thank Korea National Herbal Cosmeceutical Material Bank supported by KOSEF for providing some materials for these studies. These studies were supported by grants awarded by the Regional Innovation Center Program (Research Center for Biomedical Resources of Oriental Medicine at Daegu Haany University) of the Ministry of Knowledge Economy and by the National Heart, Lung, and Blood Institute of the National Institute of Health HL 68571 and HL 62565 to ARR.

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Figure 1. The barrier permeability effect of thrombin on endothelial cells

(A) Effect of indicated concentrations of thrombin on permeability in primary HUVECs were monitored as described under “Materials and Methods”. (B) The same as (A) except that permeability was monitored at an optimal concentration of thrombin (50 pM) in either the absence or presence of blocking (B) or non-blocking (NB) anti-PAR-1 antibodies. (C and D) The same as (A and B) except that the cell type was primary HPAECs.

Figure 2. Analysis of the TNF- α -mediated adhesion of leukocytes to endothelial cells

TNF- α -mediated (10 ng/mL for 4 hours) adhesion of freshly isolated neutrophils to primary HUVECs was analyzed after treating monolayers with indicated concentrations of thrombin in the absence (A) or presence of blocking or nonblocking anti-PAR-1 antibodies (B) as described under "Materials and Methods". (C and D) The same as (A and B) except that the cell type was primary HPAECs.

Figure 3. Analysis of the TNF- α -mediated transendothelial migration (TEM) of leukocytes through endothelial cells

TNF- α -mediated (10 ng/mL for 4 hours) TEM of freshly isolated neutrophils to primary HUVECs was analyzed after treating monolayers with indicated concentrations of thrombin in the absence (A) or presence of function-blocking or non-blocking anti-PAR-1 antibodies (B) as described under "Materials and Methods". (C and D) The same as (A and B) except that the cell type was primary HPAECs.

Figure 4. Analysis of the expression of TNF- α -mediated cell adhesion molecules (CAM) on endothelial cells and the inhibitory effect of low concentrations of thrombin

Confluent monolayers of HUVECs were treated with different concentrations of thrombin for 4 hours followed by analysis of the expression of VCAM-1 (whiter bar), ICAM-1 (grey bar) and E-selectin (black bar) in response to TNF- α for 4 hours by an ELISA in the absence (A) or presence of blocking or non-blocking ant-PAR-1 antibodies (B) as described under "Materials and Methods". (C and D) The same as (A and B) except that cell type was primary HPAECs.

Figure 5. The effect of PAR-1 agonist peptide on barrier permeability in endothelial cells
(A) The effect of increasing concentrations of PAR-1 agonist peptide, TFLLRN, (x-axis) on the permeability in HUVECs was monitored as described under “Materials and Methods”.
(B) The same as (A) except that the cell type was primary HPAECs.

Figure 6. The effect of PI3 kinase inhibitor (LY-294002) on antiinflammatory activities of low concentrations of thrombin in endothelial cells

(A) Endothelial cells were preincubated with LY-294002 (10 μ M) followed by monitoring the permeability in the presence of 50 pM thrombin in HUVECs (white bar) or 75 pM on HPAECs (Black bar) as described under “Materials and Methods”. (B) The same as (A) except that neutrophil adhesion to endothelial cells was monitored as described under “Materials and Methods”. (C) The same as (A) except that TEM was measured as described under “Materials and Methods”.