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Adenosine regulates the proinflammatory signaling function of thrombin in endothelial cells

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Abstract

The plasma level of the regulatory metabolite adenosine increases during the activation of coagulation and inflammation. Here we investigated the effect of adenosine on modulation of thrombin-mediated proinflammatory responses in HUVECs. We found that adenosine inhibits the barrier-disruptive effect of thrombin in HUVECs by a concentration-dependent manner. Analysis of cell surface expression of adenosine receptors revealed that A_{2A} and A_{2B} are expressed at the highest level among the four receptor subtypes (A_{2B}>A_{2A}>A₁>A₃) on HUVECs. The barrier-protective effect of adenosine in response to thrombin was recapitulated by the A_{2A} specific agonist, CGS 21680, and abrogated both by the siRNA knockdown of the A_{2A} receptor and by the A_{2A}-specific antagonists, ZM-241385 and SCH-58261. The thrombin-induced RhoA activation and its membrane translocation were both inhibited by adenosine in a cAMP-dependent manner, providing a molecular mechanism through which adenosine exerts a barrier-protective function. Adenosine also inhibited thrombin-mediated activation of NF-κB and decreased adhesion of monocytic THP-1 cells to stimulated HUVECs via down-regulation of expression of cell surface adhesion molecules, VCAM-1, ICAM-1 and E-selectin. Moreover, adenosine inhibited thrombin-induced elevated expression of proinflammatory cytokines, IL-6 and HMGB-1; and chemokines, MCP-1, CXCL-1 and CXCL-3. Taken together, these results suggest that adenosine may inhibit thrombin-mediated proinflammatory signaling responses, thereby protecting the endothelium from injury during activation of coagulation and inflammation.

Keywords

Adenosine; Adenosine receptor; Endothelium; Permeability; Thrombin

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Authorship contributions

S.M.H. designed experiments, performed research, analyzed data and wrote the manuscript; P.D. performed research and conducted statistical analysis; A.R.R. revised the manuscript, conceived of and directed the project.

Conflict-of-interest disclosure

The authors declare no competing financial interest.

Introduction

Thrombin, a multifunctional serine protease in plasma, is involved in regulation of numerous pathophysiological processes related to coagulation and inflammation (Esmon, 2013; Riewald et al., 2002; Mosnier et al., 2007; Joyce et al., 2001). It possesses both procoagulant and anticoagulant as well as proinflammatory properties (Esmon, 2013; Riewald et al., 2002; Mosnier et al., 2007). The latter function of thrombin has been extensively studied in cellular models and it has been established that the activation of protease-activated receptors (PAR) 1 and 4 by thrombin can initiate proinflammatory signaling responses in various cell types (Coughlin, 2005; Bae et al., 2007). The activation of PAR1 and PAR4 on vascular endothelial cells by thrombin has been shown to up-regulate expression of cell adhesion molecules and secretion of an array of proinflammatory cytokines and chemokines that are regulated by nuclear factor (NF)- κ B (Joyce et al., 2001; Rahman et al., 1999; Ruf et al., 2003). Thus, it has been demonstrated that treatment of human umbilical vein endothelial cells (HUVECs) with thrombin causes an increase in vascular permeability, over-expression of VCAM-1, I-CAM-1 and E-selectin, and enhanced adherence of leukocytes to thrombin-stimulated cells (Rahman et al., 1999; Ruf et al., 2003). Although thrombin can trigger a negative feed-back loop to inhibit its own production through the protein C anticoagulant pathway (Esmon, 2013), the mechanisms that may be involved in modulation of the receptor-mediated proinflammatory signaling function of thrombin under physiological conditions have not been fully investigated.

Adenosine is an endogenous purine nucleoside present in every cell of the human body (Eltschig et al., 2012). It plays an important role in various pathophysiological processes including angiogenesis, cardiovascular homeostasis, ischemic pre- and post conditioning and inflammation (Adair, 2004; Ely et al., 1992; Linden, 2006). The signaling effect of adenosine on cells is mediated through four different adenosine receptors, A₁, A_{2A}, A_{2B} and A₃ all of which belong to the G-protein coupled family of receptors (Chen et al., 2013). Both A₁ and A₃ can couple to inhibitory G-proteins, G_o or G_i, thus mediating the inhibition of adenylate cyclase. Whereas the A₂ subtypes, A_{2A} and A_{2B}, can both couple to G_s, thereby increasing production of intracellular cAMP (Johnston-Cox et al., 2012). Endothelial cells are known to constitutively produce adenosine at sites of vascular injury to exert immunomodulatory and immunosuppressive effects during metabolic stress and activation of coagulation and inflammation (Hasko et al., 2004; Gunther et al., 1991). Moreover, it has been demonstrated that cultured endothelial cells, exposed to thrombin, can liberate a high proportion of their adenosine nucleotide which can be extracellularly converted to adenosine (Pearson and Gordon, 1979). Whether adenosine signaling through either one of its receptors can modulate thrombin-mediated signaling responses in vascular endothelial cells has not been investigated.

In this study, we investigated this question by analyzing the modulatory effect of adenosine on thrombin-mediated signaling responses in HUVECs. Results suggest that adenosine activation of the A_{2A} receptor, but not other receptor subtypes, inhibits thrombin-induced barrier destabilization in HUVECs. Attenuation of thrombin-induced RhoA activation (Rho-GTP) by adenosine was found to account for the molecular mechanism through which adenosine is able to reverse the barrier-disruptive function of thrombin. Results further

demonstrate that adenosine inhibits activation of NF- κ B in thrombin-stimulated endothelial cells, thereby down-regulating cell surface expression of cell adhesion molecules ICAM-1, VCAM-1 and E-selectin. Consistent with its potent modulatory effect, adenosine also effectively inhibited thrombin-stimulated expression and secretion of proinflammatory cytokines and chemokines, suggesting that adenosine may play a cytoprotective role during activation of coagulation and inflammation at vascular injury sites under various pathophysiological conditions.

Materials and Methods

All adenosine receptor specific antagonists: DPCPX (A_1 subtype specific), SCH-58261 and ZM-241385 (A_{2A} -specific), and MRS 1754 (A_{2B} -specific) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The A_{2A} -specific agonist, CGS 21680, was purchased from EMD Millipore (Billerica, MA, USA). The cAMP inhibitor, RP-cAMP, and adenosine siRNAs were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased (Cambrex Bio Science Inc. Charles City, IA) and grown according to the manufacturer's instruction. Immortalized HUVECs (EA.hy926) was kindly provided by Dr. CJ Edgell (University of North Carolina at Chapel Hill, NC, USA) and maintained as described (Bae et al., 2007). The human monocytic leukemia cell line, THP-1 (ATCC, Manassas, VA), was maintained at a density of $2 \times 10^5 - 1 \times 10^6$ cells mL^{-1} in RPMI-1640 supplemented with 10% FBS.

Permeability assays and transient transfection with siRNA

Endothelial cell permeability in response to thrombin was determined in a dual chamber model by measuring the flux of Evans blue-labeled BSA across the cell monolayer as described (Bae et al., 2007). Briefly, cells were seeded at an average density of 5×10^4 cells/well in 3- μm pore size in trans-well plates (Corning Inc., Corning, NY) and grown until confluence (4–6 days). Cells at the upper chamber were treated with increasing concentrations of adenosine for 30 min followed by incubation with 20 nM thrombin for 20 min in serum-free medium containing 0.5% BSA. During adenosine receptor antagonist treatments, antagonists were added for 30 min prior to adenosine treatment. Same procedures were employed to monitor the inhibitory effect of adenosine on the barrier-disruptive effect of thrombin after transfecting cells with specific siRNAs targeting adenosine receptors as described (Bae et al., 2007). Briefly, 3.5×10^5 cells were seeded in 6-well plates and transfected with 50 pmol siRNA and 5 μL Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Following 48h incubation at 37°C in 5% CO_2 , cells were trypsinized and seeded at a density of 4×10^5 cells/well in upper chambers. The next day (24h), cells were incubated with thrombin and/or adenosine to perform the permeability assay as described above.

Real-time quantitative PCR

Total RNA was extracted using AurumTM Total RNA Mini Kit (Bio-Rad, Hercules, CA), and 1 μg of the total RNA was reverse-transcribed with the high-capacity cDNA reverse

transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was run on a LightCycler Roche 480 with the SYBR Advantage qPCR Premix (Clontech, Mountain View, CA). The amplification proceeded with an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and combined annealing/extension at 63°C for 30 s. A melt-analysis was run for all products to evaluate the specificity of the amplification. Results were normalized to expression levels of GAPDH and presented as the fold difference relative to the control group at each time point using the 2^{-C_t} method. Primer sets for individual genes (Table 1), were designed using the Primer 3 program at the Whitehead Institute for Biomedical Research/MIT Center for Genome Research at <http://www.genome.wi.mit.edu> and synthesized by IDT (Integrated DNA Technologies, Coralville, Iowa).

Western-blot analysis

Cells were treated with different stimulators as indicated and lysed with the Pierce IP lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific Pierce, Rockford, IL). The lysate clarified by centrifugation at 13,000g for 10 min at 4°C and the protein content was measured using a protein assay kit (Bio-Rad, Richmond, CA). Protein samples were boiled in SDS loading buffer, separated on a 10% SDS denaturing gel and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Immunoblots were blocked for 2h in TBST buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.5, 0.1% Tween 20) containing 5% non-fat milk. Western immunoblots were incubated with primary and secondary antibodies and developed with Enhanced Chemiluminescence reagents (Millipore, Billerica, MA). To detect the secretion of high mobility group box 1 (HMGB-1) protein, the conditioned medium from endothelial cells were concentrated by centrifugation at 2,000 rpm at 4°C for 15 min through Centricon-10 micro-concentrator filters with a 10-kDa cut off (Amicon Corp., Danvers, MA) and equal volumes of concentrated media were mixed with loading buffer and after boiling separated on a 10% SDS denaturing gel. Membrane was probed with anti-HMGB-1 antibody (Cell Signaling Technology, Beverly, MA) and proteins were visualized by SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

Cell-based ELISA

The expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells was quantified using a whole-cell ELISA as described (Bae and Rezaie, 2011). Briefly, endothelial cells were plated at a concentration of 4×10^4 cells/well in 96-well, flat-bottom, gelatin-coated plates and grown to confluence at 37°C. The cell monolayer was treated with 20 nM thrombin for 8h in the presence or absence of increasing concentrations of adenosine (10–500 μ M) and then fixed in 4% paraformaldehyde for 30 min. Nonspecific protein binding was blocked by adding 1% BSA in PBS for 1h at room temperature. Cells were washed with PBS and incubated with primary mouse anti-human VCAM-1 and ICAM-1 antibodies (Millipore, Billerica, MA) for 1h followed by incubation with the peroxidase-conjugated rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected by

measuring the optical density at 405 nm using ABTS peroxidase substrate (KPL, Gaithersburg, MD).

Cell adhesion assay

The adherence of THP-1 cells to HUVECs was evaluated using fluorescent labeling of THP-1 cells as recommended by the manufacturer (Invitrogen) as described (Bae and Rezaie, 2008). Briefly, endothelial cells were treated with thrombin in the presence and absence of increasing concentrations of adenosine for 8h. The cell monolayer was then washed and 1×10^5 THP-1 cell suspension, loaded with the fluorescent dye Vybrant DiD for 15 min, was added to cells. Cells were allowed to adhere for 15 min at 37°C and non-adherent THP-1 cells were removed by gentle washing with PBS. The fluorescence emission of adherent cells was measured at λ 665 by the microplate reader. The percentage of adherent THP-1 cells was calculated by the formula: %adherence = (adherent signal/total signal) $\times$ 100.

Subcellular fractionation and RhoA membrane translocation

Subcellular fractionation was performed as described (Bae and Rezaie, 2009). Briefly, upon treatment, cells were washed, scraped in buffer A (PBS containing 2 mM EDTA supplemented with protease inhibitor cocktail) and disrupted by brief sonication. Unbroken cells were removed by centrifugation at 850g for 10 min at 4°C. The supernatant was then centrifuged at 100,000g for 1h at 4°C (Beckman Instrument, Inc., Fullerton, CA) to separate membrane and cytosol fractions. The supernatant corresponding to the cytosolic fraction was stored and the pellet was resuspended in buffer B containing 100 mM Tris-HCl, 300 mM NaCl, 1% Triton X-100, and 0.1% SDS, 2 mM EDTA and 1 $\times$ protease inhibitor cocktail. The membrane fraction was solubilized by sonication as described above and equivalent amounts of membrane proteins were resolved on 12% SDS-PAGE gel. Western-blotting was performed to determine RhoA membrane accumulation using monoclonal anti-RhoA antibody (Cytoskeleton, Inc., Denver, CO) as described (Bae and Rezaie, 2009). Expression of RhoA in the cytosolic and β -actin in membrane fractions were also monitored as normalization controls.

Analysis of RhoA activation by pull down assay

The activation of RhoA (RhoA-GTP) was quantitated by affinity precipitation method using a glutathione S-transferase fusion protein of the Rhotekin-Rho binding domain (GST-RBD) that specifically pulls down active GTP-bound RhoA as described (Bae and Rezaie, 2009). Briefly, conditioned cells (see figure legends) were harvested with cell lysis buffer provided by the assay kit. 50 μ g of pre-cleared lysates were saved for the Western quantitation of the total RhoA. Equal amounts of lysate proteins (300 μ g) were incubated with 20 μ g of the GST-RBD fusion protein coupled to glutathione agarose beads to selectively bind to activated RhoA-GTP for the pull-down assay. Beads were washed and RhoA-GTP was eluted by boiling in 15 μ L Laemmli sample buffer. Eluted samples from the beads and 50 μ g of total cell lysates were separated on 12% SDS-PAGE and analyzed by immunoblotting using an anti-RhoA monoclonal antibody. The amount of GTP-RhoA was normalized for the total amount of RhoA present in each sample.

Statistical analysis

All values are presented as mean $\pm$ S.E. Comparison between groups was made using One-way ANOVA test followed by the Bonferroni post hoc test. $p < 0.05$ was considered to be statistically significant.

Results

Effect of adenosine on thrombin-induced hyperpermeability in endothelial cells

It has been established that thrombin disrupts the barrier permeability function of endothelial cells (Bae et al., 2007; Feistritzer and Riewald, 2005). To examine the effect of adenosine signaling in cell permeability in response to thrombin, endothelial cells were pretreated with adenosine (0–100 μ M) for 30 min prior to their activation by thrombin (20 nM for 20 min). The preincubation of endothelial cells with adenosine markedly decreased the barrier-disruptive effect of thrombin by a concentration dependent manner (Fig. 1A). Both normal and transformed HUVECs yielded identical results. A significant barrier-protective effect for adenosine could be observed at the concentration range of 10–100 μ M (Fig. 1A). This range may pathophysiologically be relevant since adenosine concentration in ischemic or inflamed tissues has been reported to reach as high as 100 μ M (Hasko and Cronstein, 2004). Since the adenosine effect is mediated through binding and activation of four adenosine receptor subtypes (Hasko and Cronstein, 2004), expression of these receptors was assessed in endothelial cells. It was found that adenosine receptor subtypes A_2 (A_{2A} and A_{2B}) have the highest expression level, A_1 exhibits a medium expression level and the expression level of A_3 subtype is negligible in HUVECs (Fig. 1B).

Protective effect of adenosine on thrombin-induced barrier dysfunction is mediated through A_{2A} receptor

To determine which adenosine receptor subtype is specifically involved in the protective effect of adenosine on thrombin-mediated barrier destabilization, endothelial cells were transiently transfected with 50 pmol siRNA targeting each specific adenosine receptor subtype. The efficiency of gene knockdown was monitored by Real-Time quantitative PCR at different time points. Results showed that specific siRNAs can significantly down-regulate expression of all four adenosine receptor subtypes in a time-dependent manner with the maximum inhibition occurring at 72h after transfection (Fig. 2A, shown for the A_{2A} subtype only). The siRNA knockdown of the A_{2A} adenosine receptor effectively suppressed the barrier-protective effect of adenosine in thrombin-stimulated cells (Fig. 2B). The abrogation of adenosine-induced barrier-protective effect by the siRNA knockdown of A_{2A} receptor was specific since neither scrambled siRNA nor siRNAs targeting other adenosine receptor subtypes showed any inhibitory effect (Fig. 2B).

To provide further support for a barrier-protective role for the A_{2A} receptor signaling in response to thrombin, we examined the effect of selective adenosine receptor antagonists on thrombin-induced barrier-disruptive function in endothelial cells. In this case, cell monolayers were pre-exposed to adenosine receptors antagonists (100 nM) for 30 min prior to incubation with adenosine and stimulation by thrombin. Consistent with receptor knockdown results, the A_{2A} -specific antagonists, ZM 241385 and SCH 58261, but not other

adenosine receptor antagonists, abrogated the barrier-protective effect of adenosine in response to thrombin (Fig. 2C). Consistent with these results, pharmacologic activation of the A_{2A} adenosine receptor with the A_{2A} specific agonist, CGS 21680, inhibited thrombin-induced hyper-permeability in a concentration dependent manner with the maximal effect occurring at 50 nM (Fig. 2D). Taken together, these results suggest that the barrier-protective effect of adenosine in response to thrombin is mediated through the activation of A_{2A} adenosine receptor in endothelial cells.

Effect of adenosine on thrombin-induced RhoA activation and membrane translocation

We and others have demonstrated that thrombin rapidly and transiently modulates activation of Rho GTPases in endothelial cells (Bae and Rezaie, 2009; Spindler et al., 2010; Beckers et al., 2010). Since Rho GTPases are involved in regulation of vascular barrier functions and thrombin exerts a barrier-disruptive effect through activation of RhoA (Spindler et al., 2010; Beckers et al., 2010), we decided to determine whether the barrier-protective effect of adenosine is associated with attenuation of RhoA activation by thrombin. The amount of RhoA-GTP was greatly enhanced in thrombin-activated endothelial cells with the maximal effect occurring after 5 min of stimulation (Fig. 3A). By contrast, pretreatment of cells with adenosine prior to stimulation by thrombin resulted in a significant decrease in the RhoA-GTP level (Fig. 3A), suggesting that inhibition of thrombin-mediated RhoA activation by adenosine may account for the molecular mechanism through which adenosine reverts the barrier-disruptive effect of thrombin. The evaluation of the inhibitory effect of adenosine on thrombin-induced RhoA activation in the presence of the antagonist of the cAMP pathway, Rp-cAMP, indicated that the protective effect of adenosine is mediated through synthesis of cAMP (Fig. 3B).

Since activation of RhoA requires guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP in RhoA and because GEFs for Rho proteins are widely assumed to be membrane-associated (Ridley, 2001), we decided to analyze the membrane translocation of RhoA as an indirect indication of RhoA activation. In agreement with results presented above, adenosine significantly decreased thrombin-stimulated RhoA membrane translocation (Fig. 3C). The cytosolic expression of RhoA and β -actin in membrane fractions were also monitored as normalization controls (Fig. 3C). These results suggest that adenosine inhibits both the activation of RhoA (Rho-GTP) and its membrane translocation in endothelial cells in response to thrombin.

Inhibitory effects of adenosine on thrombin-induced expression of cell adhesion molecules, adhesion of THP-1 cells and activation of NF- κ B

To further investigate inhibitory functions of adenosine on proinflammatory signaling by thrombin, the effect of adenosine on the protein and/or RNA level of cell adhesion molecules was evaluated in endothelial cells in response to thrombin. Results presented in Fig. 4A demonstrate that thrombin dramatically up-regulates expression of both ICAM-1 and VCAM-1 and adenosine significantly down-regulates the cell surface expression of both cell adhesion molecules. Pharmacological activation of the A_{2A} adenosine receptor with CGS 21680 decreased the expression of both ICAM-1 and VCAM-1 in thrombin stimulated endothelial cells further indicating that A_{2A} is responsible for the protective effect of

adenosine in response to thrombin (Fig. 4B). Adenosine also inhibited the thrombin-mediated elevated expression of E-selectin mRNA in endothelial cells (Fig. 4C). The modulatory effect of adenosine on expression of adhesion molecules correlated with the inhibitory function of adenosine on binding of THP-1 cells to thrombin-stimulated endothelial cells (Fig. 4D).

Phosphorylation of the transcription factor p65 is a known mechanism through which thrombin activates NF- κ B, thereby enhancing expression of cell adhesion molecules (Anwar et al., 2004). In agreement with previous results, thrombin activated NF- κ B through phosphorylation of p65 (Ser 536) and adenosine effectively inhibited this function of thrombin in a concentration dependent manner (Fig. 4E).

Effect of adenosine on thrombin-induced expression and secretion of proinflammatory cytokines and chemokines

HMGB-1 is a late-acting proinflammatory nuclear cytokine which upon secretion to circulation triggers activation of the endothelium and leukocytes (Lotze, 2005). We investigated the effect of thrombin on HMGB-1 secretion in the presence and absence of adenosine in endothelial cells. Interestingly, thrombin significantly stimulated secretion of HMGB-1 to the cell culture supernatant and this effect was completely abrogated by adenosine (Fig. 5A). Further studies showed that adenosine also down-regulates RNA expression levels of the early-acting cytokine, IL-6, as well as the monocyte chemokines, CXCL-3 and MCP-1 and the neutrophil chemokine CXCL-1 in thrombin-stimulated endothelial cells by a concentration dependent manner (Fig. 5B and C).

Discussion

In this study we demonstrate for the first time that adenosine signaling inhibits proinflammatory signaling function of thrombin in vascular endothelial cells. Our results indicate that adenosine inhibits proinflammatory functions of thrombin specifically through the A_{2A} receptor signaling. Stimulation of A_{2A} receptor by adenosine prevents the barrier-disruptive function of thrombin and inhibits thrombin-induced activation of RhoA (Rho-GTP) in endothelial cells. Adenosine signaling also inhibits thrombin-mediated up-regulation of proinflammatory cytokines and chemokines as well as the elevated expression of cell adhesion molecules ICAM-1, VCAM-1 and E-selectin through inhibition of activation of the NF- κ B pathway. Consistent with its role in down-regulation of cell adhesion molecules, adenosine also inhibits adhesion of the monocytic THP-1 cells to thrombin-stimulated endothelial cells. Extracellular HMGB-1 is a late-acting nuclear cytokine known for its potent proinflammatory role in various inflammatory diseases (Lotze, 2005). It was interesting to note that adenosine inhibits thrombin-mediated secretion of HMGB-1 in endothelial cells as well. HMGB-1 is known to induce proinflammatory signaling through interaction with pattern recognition receptors including Toll-like receptors (TLRs). Thus, adenosine can modulate signal transduction pathways through pattern recognition receptors in endothelial cells. This hypothesis is consistent with previous data that adenosine signaling elicits protective signaling responses in immune cells stimulated with various agonists of TLRs (Hasko and Cronstein, 2004). Further studies will be required

to determine whether adenosine can also modulate the cell surface expression of pattern recognition receptors in endothelial cells. Taken together, results presented in this manuscript suggest that adenosine signaling through the A_{2A} receptor can down-regulate thrombin-mediated proinflammatory response during activation of the coagulation cascade.

Inflammation and coagulation are closely linked processes and activation of endothelial cells plays a critical role in driving both biological systems. An injury to vasculature triggers activation of the clotting cascade and generation of thrombin which in turn activates platelets, releasing numerous metabolites, cytokines and chemokines into circulation. The released inflammatory molecules can activate endothelium, up-regulate thrombin generation and facilitate recruitment of innate immune cells to the site of vascular injury. Activated endothelial cells express cell surface adhesion molecules to support transmigration of immune cells to injured tissues. Thus, activation of coagulation amplifies inflammation which in turn amplifies more thrombin generation and cytokine secretion. This disruptive amplification cycle must be tightly regulated in order to prevent activated inflammatory cells and various proinflammatory mediators from damaging and compromising the integrity of the vasculature. Our results suggest that adenosine signaling may contribute one such regulatory mechanism which down-regulates the propagation of proinflammatory responses in endothelial cells. Both neutrophils and endothelial cells are known to store high levels of adenosine which can be released at sites of injury during activation of coagulation and inflammation (Hasko and Cronstein, 2004). Moreover, thrombin-mediated platelet activation releases ADP which can be dephosphorylated to adenosine (Ralevic and Burnstock, 1998), thereby not only down-regulating ADP-mediated platelet activation and further thrombin generation, but also elevating the concentration of the protective metabolite at injured sites to as high as 100 μ M (Hasko and Cronstein, 2004). Our results suggest that adenosine signaling through A_{2A} receptor constitutes an important regulatory mechanism which can protect vasculature from injury by inflammatory mediators. The significance of this regulatory mechanism becomes even more evident when one notes that thrombin has been reported to elicit potent proinflammatory responses through activation of both PAR1 and PAR4 in endothelial cells. Studies in cellular models have indicated that a thrombin concentration of as low as 0.5 nM can disrupt the barrier permeability function, mediate activation of NF- κ B and induce expression of proinflammatory molecules whose transcription is regulated by this nuclear transcription factor. Nevertheless, based on the results presented above, assuming that the concentration of adenosine can approach 10 μ M or higher, we hypothesize that adenosine signaling under in vivo conditions can modulate these proinflammatory responses of thrombin, thereby protecting the integrity of the endothelium and stabilizing its barrier permeability function to prevent excess edema formation at vascular injury sites.

Inhibition of proinflammatory thrombin signaling by adenosine may not be the only mechanism through which thrombin signaling is modulated in vascular endothelial cells. We have also demonstrated that the occupancy of endothelial protein C receptor (EPCR) by protein C also alters the PAR1-dependent signaling specificity of thrombin from a proinflammatory to an antiinflammatory response (Bae et al., 2007, Bae and Rezaie, 2011, 2008, 2009). Thus, we showed that pretreatment of HUVECs with the protein C zymogen (to occupy EPCR with its ligand) prior to stimulation by thrombin yielded a barrier-

protective response through the protease cleaving the PAR1 receptor. The protective EPCR- and A₂-adenosine receptor-dependent signaling in endothelial cells can constitute safe-guard mechanisms which ensure that proinflammatory signaling responses during the activation of coagulation do not disrupt the integrity of the vasculature.

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Figure 1.

Effect of adenosine on thrombin-induced permeability and analysis of basal expression level of adenosine receptors in endothelial cells. (A) Endothelial cells were incubated with thrombin (Thr) 20 nM for 20 min with or without prior incubation with indicated concentrations of adenosine (Ad) for 30 min followed by measuring permeability as described in Methods. (B) Basal expression level of adenosine receptors was determined via Real-time quantitative PCR in unstimulated endothelial cells. All results are means $\pm$ SE of three different experiments. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$

Figure 2.

Adenosine inhibits the barrier-disruptive effect of thrombin through activation of adenosine A_{2A} receptor in endothelial cells. (A) Cells were transiently transfected with A_{2A} siRNA for the indicated period before analysis of the mRNA expression level by Real-time quantitative PCR using a specific primer. (B) Endothelial cells, transiently transfected with control or adenosine receptors siRNAs, were pre-incubated for 30 min with adenosine (100 μ M) and then stimulated with 20 nM thrombin for 20 min. (C) Confluent endothelial cells in a dual chamber system were pre-treated with adenosine receptor antagonists (100 nM) for 30 min prior to incubation with adenosine (100 μ M) and induction of permeability with 20 nM thrombin for 20 min. (D) Cells were pre-treated with increasing concentrations of A_{2A} specific agonist, CGS 21680, for 30 min followed by stimulation with thrombin 20 nM for 20 min. Each bar represents mean $\pm$ SE of three independent measurements. * $p < 0.05$; ** $p < 0.01$.

Figure 3.

Adenosine inhibits thrombin-induced RhoA activation and membrane translocation in endothelial cells. (A) Western-blot analysis of the effect of thrombin (20 nM for 5 min) on RhoA activation (Rho-GTP) was monitored in the presence or absence of adenosine as described in Methods. Lane 1, not treated control; lanes 2–4, cells were treated with thrombin at different times; lanes 5–7, cells were incubated with increasing concentrations of adenosine for 3h followed by addition of thrombin (20 nM for 5 min). (B) Cells were pre-treated with or without increasing concentrations of the cAMP antagonist, Rp-cAMP, for 1h followed by incubation with adenosine (100 μM) for 3h and stimulation with thrombin (20 nM) for 5 min. (C) The same as A except that instead of RhoA pull-down, translocation of RhoA to the membrane was studied as described in Methods. All values are means $\pm$ SE of three independent experiments. * $p < 0.05$; ** $p < 0.01$

Figure 4.

Adenosine inhibits thrombin-induced up-regulation of adhesion molecules, monocyte THP-1 adhesion and NF- κ B activation in endothelial cells. (A) Cells were treated with either thrombin (20 nM) or adenosine alone (lane 2–4) or with combination of both (lane 5–7) for 8h followed by analysis of the expression of VCAM-1 and ICAM-1 as described in Methods. (B) The same as (A) except that the effect of increasing concentrations of the A_{2A} agonist, CGS 21680, on the thrombin-induced expression of adhesion molecules was analyzed. (C) The effect of adenosine on thrombin-mediated increase in E-selectin mRNA level was determined by Real-time PCR. Lane 1, not treated control; lane 2, adenosine (100 μM for 3h); lane 3–4, thrombin (20 nM) at 1h and 3h respectively; lane 5–6, cells were treated with 10 or 100 μM adenosine before stimulation with 20 nM thrombin for 3h. (D) The same as (A) except that the effect of the increasing concentrations of adenosine on the adhesion of monocyte THP-1 cells to thrombin-stimulated endothelial cells was analyzed. (E) The effect of adenosine on thrombin-mediated (20 nM for 1h) activation of NF- κ B was analyzed by Western blotting using specific antibodies as described in Methods. All results are means $\pm$ SE of three different experiments. * p < 0.05; ** p < 0.01

Figure 5.

Adenosine inhibits thrombin-induced expression and secretion of proinflammatory cytokines and chemokines in endothelial cells. (A) Cells were pretreated with indicated concentrations of adenosine for 3h followed by incubation with thrombin (20 nM). The secretion of HMGB-1 to the supernatant was measured as described in Methods. (B) Real-time quantitative PCR analysis of IL-6, CXCL-1 and CXCL-3 genes expression in endothelial cells. Lane 1, treated control; lane 2, adenosine (100 μ M for 3h) only; lane 3–4, thrombin 20 nM at 3h and 1h respectively; lane 5–6, cells were treated with 10 or 100 μ M adenosine (for 3h) followed by stimulation with thrombin (20 nM for 1h). (C) Effect of adenosine on thrombin-induced increase in MCP-1 expression was analyzed in endothelial cells. Cells were pretreated with increasing concentration of adenosine for 3h followed by stimulation with thrombin (20 nM for 2h). All results are means $\pm$ SE of three different experiments. * $p < 0.05$; ** $p < 0.01$

Table 1

Primer sequences employed in Real-time quantitative PCR assays. ADOR, adenosine receptor

Gene	Primer Sequence (5'-3')
GAPDH	CTGGGCTACACTGAGCACC AAGTGGTCGTTGAGGGCAATG
ADOR A ₁	CAAGATCCCTCTCCGGTACAA GCCAAACATAGGGGTCAGTCC
ADOR A _{2A}	CTGGCTGCCCCCTACACATC TCACAACCGAATTGGTGTGGG
ADOR A _{2B}	CTGTCACATGCCAATTCAGTTG GCCTGACCATTCCCACTCTTG
ADOR A ₃	GTGCTGGTCATGCCTTTGG CGTGGGTAAAGATAAGCAGTAGG
E-selectin	CAGCAAAGGTACACACACCTG CAGACCCACACATTGTTGACTT
IL-6	CCTGAACCTTCCAAAGATGGC TTCACCAGGCAAGTCTCCTCA
CXCL-1	AGTGACAAATCCAACCTGACC GATGCTCAAACACATTAGGC
CXCL-3	CCAAACCGAAGTCATAGCCAC TGCTCCCCTTGTTTCAGTATCT
MCP-1	CAGCCAGATGCAATCAATGCC TGGAATCCTGAACCCACTTCT