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Role of Protein Kinase C ζ in Thrombin-induced RhoA Activation and Interendothelial Gap Formation of Human Dermal Microvessel Endothelial Cell Monolayers

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Abstract

We studied the potential involvement of the Ca²⁺-independent atypical protein kinase C isoform PKC ζ in mediating the thrombin-induced increase in endothelial permeability. Studies were done using human dermal microvessel endothelial cells (HMEC), which we showed constitutively expressed PKC ζ . We quantified the patency of inter-endothelial junctions (IEJs) and endothelial barrier function by measuring transendothelial electrical resistance (TER) in confluent HMEC monolayers. In control monolayers, thrombin decreased TER by ~50%, indicating thrombin-dependent opening of IEJs. Thrombin also elicited increases in cytosolic Ca²⁺ concentration [Ca²⁺]_i, actin stress fiber formation, and myosin light chain (MLC) phosphorylation. Pan-PKC inhibitors, calphostin C and chelerythrine, abrogated these responses. Thrombin also decreased TER after depletion of conventional and novel Ca²⁺-dependent PKC isoforms using phorbol 12-myristate 13-acetate (PMA). In these PMA-treated cells, thrombin induced inter-endothelial gap formation, MLC phosphorylation, and actin stress fiber formation, but failed to increase [Ca²⁺]_i. Inhibition of PKC ζ activation using the PKC ζ pseudosubstrate peptide (PSI), depletion of PKC ζ protein with siRNA, and competitive inhibition of PKC ζ activity using dominant-negative (dn) PKC ζ mutant all prevented the thrombin-induced decrease in TER and MLC phosphorylation. Expression of dn-PKC ζ also inhibited thrombin-induced RhoA activation. These findings reveal a novel Ca²⁺-independent, PKC ζ -dependent mechanism of thrombin-induced increase in endothelial permeability. The results raise the possibility that inhibition of PKC ζ may be a novel drug target for thrombin-induced inflammatory hyperpermeability.

Keywords

thrombin; atypical PKC; PKC ζ ; endothelial cell retraction; RhoA activation

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Introduction

Capillary permeability to plasma proteins is normally kept low by the restrictive nature of the endothelial junctional barrier. Inflammatory mediators such as thrombin increase endothelial permeability by stimulating endothelial cell retraction and formation of inter-endothelial gaps (Garcia et al., 1986; Garcia et al., 1996; Lum and Malik, 1994; Moy et al., 1996; Rabiet et al., 1996). The resultant increase in paracellular permeability to albumin leads to protein-rich edema, the underlying cause of several pathological conditions such as Acute Respiratory Distress Syndrome (ARDS). Thrombin increases endothelial permeability by activating specific signal transduction pathways (for reviews, see Mehta and Malik, 2006; Minshall and Malik, 2006; Vandenbroucke et al., 2008). The pathways involved are generally assumed to be Ca^{2+} -dependent based on evidence that the rise in cytosolic free Ca^{2+} is a crucial determinant of increased endothelial permeability (Lum et al., 1989; Tiruppathi et al., 2002). Thrombin has also been shown to induce a Ca^{2+} -dependent phosphorylation of substrate proteins by protein kinase α (PKC α) and myosin light chain kinase (MLCK), both of which are involved in the mechanism of increased endothelial permeability (Essler et al., 1998; Garcia et al., 1996; Lum and Malik, 1994; Lynch et al., 1990; Mehta et al., 2001; Moy et al., 1996; Nguyen et al., 1997; Rabiet et al., 1996; Sandoval et al., 2001; Tiruppathi et al., 1992a; Tiruppathi et al., 1992b; van Nieuw Amerongen et al., 1998; Vouret-Craviari et al., 1998).

Studies have also shown the importance of RhoA signaling in endothelial cell retraction (van Nieuw Amerongen et al., 1998; Vouret-Craviari et al., 1998). Mehta et al. (2001) showed that thrombin-induced phosphorylation of Rho-GDI (guanine nucleotide dissociation inhibitor) by PKC α was required for RhoA activation and decreased TER in human umbilical vein endothelial cells (HUVECs). These observations indicate that PKC α regulation of RhoA activation can signal increased endothelial permeability. In addition, PKC α -mediated phosphorylation of p115RhoGEF induced by thrombin was shown to stimulate RhoA activation in HUVECs (Holinstat et al., 2003). Thus, PKC α and RhoA acting in concert may be required for activation of the endothelial contractile machinery via stimulation of G-protein coupled pathways (Holinstat et al., 2003; McLaughlin et al., 2005; Mehta et al., 2001; Vanhauwe et al., 2002).

Since Ca^{2+} signaling is critical in the mechanism of thrombin-induced endothelial cell contraction (for review, see Tiruppathi et al., 2003), studies have addressed the role of specific Ca^{2+} channels in endothelial cells responsible for Ca^{2+} signaling. Tiruppathi et al., (2002) demonstrated the essential role of capacitive Ca^{2+} entry via transient receptor potential channel 4 (TRPC4). However, the increase in $[\text{Ca}^{2+}]_i$ which follows thrombin stimulation of endothelial cells is brief (lasting 2-3 min; Lum et al., 1992) but the increase in permeability persists for 2 hours (Tiruppathi et al., 1992a; Tiruppathi et al., 1992b). These findings raise the possibility that Ca^{2+} -independent mechanisms may sustain the endothelial contractile response. In the present study, we addressed the possible role of the atypical PKC isoform, PKC ζ , which does not require Ca^{2+} for activation (Standaert et al., 1997), in signaling increased endothelial permeability. Using an endothelial cell line that exhibits robust expression of PKC ζ , human dermal microvessel endothelial cells (HMEC), we observed that thrombin-induced endothelial retraction was dependent on PKC ζ activation. In addition, we found that activation of PKC ζ allows RhoA/ROCK activation, which resulted in increased actin stress fiber formation and increased endothelial permeability. We propose that a PKC ζ -dependent mechanism of increased endothelial permeability is engaged in the absence Ca^{2+} signaling. Thus, PKC ζ activation may enable the endothelial permeability response to be sustained, raising the possibility that PKC ζ may be a critical therapeutic target in inflammation.

Materials and Methods

Materials

Myristoylated PKC ζ pseudosubstrate inhibitor (PSI) peptide (Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH) was obtained from Biosource International (Camarillo, CA). Y27632 and rottlerin were from Calbiochem-Novabiochem (San Diego, CA). Human α -thrombin was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Antibodies were from the following sources: anti-phospho-Thr410-PKC ζ pAb (Cell Signaling, Beverly, MA); anti-MLC, anti-PKC ζ , -PKC α , -PKC δ , and anti-Rho pAb (Santa Cruz Biotechnology, Santa Cruz, CA); anti-diphospho-MLC pAb (Dr. J.G.N. Garcia, University of Illinois at Chicago, Chicago, IL). DAPI (4',6-diamidino-2-phenylindole, dihydrochloride), Alexa Fluor-conjugated secondary Abs, phalloidin, and ProLong antifade mounting medium were purchased from Molecular Probes/Invitrogen, Eugene, OR). PKC ζ siRNA was from Santa Cruz Biotechnology (Santa Cruz, CA) and PKC α siRNA was purchased from Dharmacon (Lafayette, CO). The mismatch (MM) control (5' UAAGGAGCCACGAGCGGUA UU 3') was a custom design.

Endothelial Cell Culture

Human dermal microvessel endothelial cells (HMEC; passage 17-21) were provided by Dr. E. Ades, Center for Disease Control (Atlanta, GA). HMEC were grown in endothelial basal medium MCDB-131 made 0.7% FBS, 10 ng/ml epidermal growth factor, 10 mM glutamine, and 1 μ g/ml hydrocortisone, 50 U/ml penicillin, and 50 μ g/ml streptomycin as described (Ades et al., 1992). We identified three PKC isoforms in HMEC monolayers: α (conventional), δ (novel), and ζ (atypical). Western blot analysis of cell lysates, while not quantitative, at least suggests that PKC ζ is relatively abundant in HMEC.

Transfection Studies

HMEC were transfected with PKC ζ siRNA, PKC α siRNA or a mismatch (MM) control siRNA using Dharmafect-1 (Dharmacon; Lafayette, CO) according to the protocol, or with PKC α and PKC ζ sense or anti-sense oligonucleotides as described (Rahman et al., 2000) using 1.25 μ g/ml oligonucleotide and 5 μ g/ml Superfect (Qiagen, Valencia, CA) per 100 mm dish. After 24 hr, cells were transferred onto No. 1 glass coverslips or gold plated impedance electrodes (Tiruppathi et al., 1992a) in media, and left for an additional 48 hr. Dn-PKC ζ mutant in pcDNA3.1 (provided by Dr. J.W. Soh; Columbia University, New York, NY) or dn-Rho mutant (N19; provided by Dr. T. Kozasa, University of Illinois at Chicago) were transfected using Superfect transfection reagent (Qiagen) as described by the manufacturer. Co-transfection with an equal concentration of plasmid pGREEN LANTERN-1 (Gibco/Invitrogen, Carlsbad, CA) was performed to identify transfected cells (Minshall et al., 2000). dn-PKC ζ adenovirus and β -galactosidase adenovirus were generously provided by Dr. V. Natarajan, University of Illinois at Chicago, Chicago, IL and used as described (Javaid et al., 2003).

Immunocytochemistry

Cells ($\sim 1 \times 10^5$) seeded onto 12 mm diameter coverslips were grown until confluent. Cells were washed and incubated 4 hr in phenol red-free (PRF) and serum-free DMEM. The cells were stimulated with 1 or 5 U/ml α -thrombin (10 or 50 nM), fixed 20 min in Hank's balanced salt solution (HBSS) plus 10 mM HEPES and 4% paraformaldehyde, and rinsed with 100 mM glycine in HBSS (3 $\times$ 7 min each) to quench free aldehyde groups. The cells were then washed 3 $\times$ in HBSS and pre-incubated 30 min in HBSS plus 0.2% BSA, 5% goat serum, 0.1% Triton-X100, and 0.01% NaN₃ (blocking buffer). Rabbit polyclonal anti-diphospho-MLC IgG (1:200) was diluted in pre-incubation buffer and incubated overnight at

4°C. Coverslips were washed 3× with HBSS plus 0.2% BSA and 0.01% NaN₃, pre-incubated 30 min in blocking buffer, and incubated with 4 µg/ml goat anti-rabbit secondary Ab (Alexa 568 conjugate) for 2 hr at room temperature (Minshall et al., 2000). In other experiments, coverslips were stained with DAPI (1 µg/ml) and 1:40 dilution (7.5 U/ml) of Alexa 488 phalloidin conjugates to label the nucleus and polymerized actin filaments, respectively. Washed coverslips were mounted using ProLong antifade mounting medium.

Confocal Microscopy

Fluorescent images were acquired with a Zeiss LSM 510 confocal microscope (University of Illinois at Chicago Research Resources Center) using a 63× NeoFluor water immersion objective, Omnichrome Ar/Kr laser and Interprise UV laser. The color images represent 1024×1024 pixel scans obtained with 568/488/364 nm excitation laser lines and LP 585/BP505-550/BP385-470 nm emission filters for Alexa-568 and 488 conjugates and DAPI; the z-axis resolution (optical section thickness < 0.5 µm) was established by setting the pinhole to achieve 1 Airy unit.

Transendothelial Electrical Resistance Measurement

Thrombin-induced endothelial cell retraction was measured by the method described by Tiruppathi et al. (1992a). Endothelial cells seeded on the gelatin-coated gold electrodes ($4.9 \times 10^{-4} \text{ cm}^2$) were grown to confluence. The smaller electrode and larger counter electrode were then connected to a phase-sensitive lock-in amplifier to monitor the voltage. A constant current of 1 µA was supplied by a 1 V, 4000 Hz AC signal connected serially to a 1 MΩ resistor between the smaller electrode and the larger counter electrode. Before each experiment, the confluent endothelial monolayers were incubated in serum-free medium for 2 hr, pretreated with inhibitors for 30 min, and then the thrombin-induced change in resistance of the endothelial monolayers was measured. Calphostin C treated cells were not exposed to light (Tiruppathi et al., 1992a). The data are presented as a change in the resistive portion of electrical impedance normalized to its value at time zero (Ellis et al., 1999; Sandoval et al., 2001; Tiruppathi et al., 1992a).

Cytosolic Ca²⁺ Measurement

The cytosolic Ca²⁺ concentration ($[\text{Ca}^{2+}]_i$) in single endothelial cells was measured as described (Sandoval et al., 2001) by fura-2 fluorescence using an Attotfluor RatioVision digital fluorescence imaging system (Atto Instruments, Rockville, MD) on a Zeiss Axiovert S100 inverted microscope with a F-Fluar 40×, 1.3 NA oil immersion objective.

Myosin Light Chain Phosphorylation

MLC phosphorylation was assayed as described (Garcia et al., 1995; Mehta et al., 2002). In brief, confluent HMEC were serum deprived for 2 hr, treated with inhibitors as noted, and stimulated with 50 nM thrombin for 0-10 min. To terminate the reaction, ice-cold 10% trichloroacetic plus 10 mM dithiothreitol was added and the cells were recovered by scraping. TCA precipitates were extracted by centrifugation/re-suspension using diethyl ether, and finally the pellet was suspended in 6.7 M urea sample buffer. Proteins were separated on glycerol-urea PAGE, transferred to nitrocellulose membranes, and MLC was detected by Western blot using rabbit polyclonal myosin light chain Ab.

RhoA Activation

Thrombin-induced RhoA activation was measured as described previously (Mehta et al., 2003) by affinity purification of RhoA-GTP with glutathione-S-transferase-tagged rhotekin binding domain (GST-RBD) conjugated beads (Cytoskeleton, Inc., Denver, CO).

Data Analysis

All values represent the mean $\pm$ SEM. Statistical comparisons were made using Student's *t* test and one-way analysis of variance using Prism (GraphPad Software, San Diego, CA); differences in mean values were considered significant at $p < 0.05$.

Results

Pan-PKC inhibitors prevent thrombin-induced barrier dysfunction in HMEC monolayers

To determine the role of protein kinase C (PKC) isoforms in the thrombin-induced increase in endothelial monolayer permeability, we measured the decrease in TER and increase in F-actin stress fiber formation in the presence of calphostin C or chelerythrine chloride. Both are pan-PKC inhibitors lacking isoform selectivity. As shown in Fig. 1A, calphostin C blocked thrombin-induced decrease in TER. As the decreased TER may be due to a loss of cell-cell tethering or actin-myosin-dependent contraction, we determined whether the increase in actin polymerization was also sensitive to these inhibitors. Chelerythrine prevented the thrombin-induced increase in actin stress fiber formation (Fig. 1B), indicating that PKC activation was required for cell contraction and increased endothelial permeability.

Depletion of conventional (c) and novel (n) PKC isoforms fails to prevent thrombin-induced increased endothelial permeability

As HMECs used in the present study expressed PKC α (conventional), δ (novel), as well as ζ (atypical) (Fig. 2A), we addressed the role of conventional and novel PKC isoforms in the permeability response. HMECs were treated for 24 hr with 500 nM PMA to deplete conventional and novel PKC isoforms as described (Rahman et al., 2001). Western blot analysis of the primary PKC isozymes expressed in HMEC, PKC α , PKC δ , and PKC ζ , was carried out in cells treated with vehicle alone or 500 nM PMA for 24 hr (Fig. 2A). We observed that PKC α was not detectable and PKC δ was reduced by $>50\%$, while PKC ζ was unaffected by PMA treatment.

Thrombin induced a transient increase in cytosolic Ca^{2+} [Ca^{2+}]_i which peaked within 30 sec and returned to baseline after 2-3 min (Fig. 2B). We also observed Ca^{2+} influx in response to extracellular application of 1 μM ionomycin, an ionophore. Following depletion of PMA-sensitive PKC isozymes, however, the thrombin-stimulated Ca^{2+} transient was reduced by 70% whereas ionomycin-induced increase in intracellular Ca^{2+} was unaffected (Fig. 2B). Thus, the thrombin-induced increase in Ca^{2+} in HMEC is likely to be dependent on classical or novel PKC isozymes. To determine changes in endothelial permeability following PMA-induced depletion of PKC α and PKC δ isoforms, TER was measured in cells pretreated with either 500 nM PMA or an inactive analogue, 4 α -phorbol 12,13-didecanoate (4 α -PDD). Thrombin induced a 40% decrease in TER in cells treated with vehicle alone, 500 nM 4 α -PDD, or 500 nM PMA (Fig. 2C), indicating that depletion of PKC α and PKC δ (conventional and novel isoforms) did not prevent thrombin-induced endothelial retraction and gap formation in HMEC. To address whether PMA exposure desensitized the cells to PMA-induced shape change, 100 nM PMA was applied to the PMA-treated cells and TER was measured. Conventional and novel PKC depletion abolished PMA-stimulated decrease in TER, whereas in control experiments (treatment with 4 α -PDD) PMA induced a sudden decrease in TER.

We also addressed the effects of pan-PKC inhibitor, chelerythrine (10 μM), on MLC phosphorylation in cells depleted of conventional and novel PKC isoforms, and stimulated with thrombin (Fig. 2D). Then, myosin extracted from whole cell lysates was separated on an 8% urea gel, transferred to nitrocellulose, and immunoblotted with anti-MLC Ab. We observed 5 bands as previously described (Garcia et al., 1995; Mehta et al., 2002) [2

unphosphorylated bands representing smooth muscle myosin (SMM) and non-muscle myosin (NMM), 1 band of monophosphorylated SMM, and 2 bands representing diphosphorylated SMM and NMM] (Fig. 2D). However, depletion of conventional and novel PKC isoforms failed to prevent thrombin-induced phosphorylation of MLC (Fig. 2D). We observed the appearance of diphospho-SMM and -NMM bands following stimulation of cells with thrombin for 1 min and prevention of thrombin-induced phosphorylation by chelerythrine. These findings suggest a role for atypical PKC in mediating thrombin-induced MLC phosphorylation.

As PKC δ was not fully depleted by PMA treatment (Fig. 2A), we also determined the effect of the PKC δ inhibitor rottlerin (10 μ M) on thrombin-induced MLC phosphorylation with and without conventional and novel PKC depletion. Both rottlerin and depletion of conventional novel PKC isoforms failed to prevent thrombin-induced MLC phosphorylation (Fig. 2E).

Activation of PKC ζ by thrombin in HMEC signals increased permeability

Since the results described above suggest a role for atypical PKCs in the mechanism of thrombin-induced increase in endothelial permeability in HMEC monolayers, we next addressed whether the prominent atypical isoform in endothelial cells, PKC ζ , is activated by thrombin. PKC ζ activation was evaluated by its membrane translocation and active site phosphorylation (Fig. 3A-3C). As shown in Fig. 3A, thrombin stimulation for 5 or 15 min resulted in translocation of PKC ζ from the cytosol to membrane, which is indicative of PKC ζ activation. Western blot analysis of phospho-Thr410- PKC ζ (pT410-PKC ζ levels in HMEC lysates treated with thrombin (Fig. 3B and 3C) showed an increase in pT410-PKC ζ within 1 min of thrombin stimulation and return towards basal by 30 min. The specificity of PKC ζ activation was shown by inhibition of phosphorylation using PKC ζ pseudosubstrate inhibitor peptide (PSI) (Fig. 3C), which was similar to the inhibition of PKC ζ phosphorylation after treatment with chelerythrine.

Since RhoA and Rho kinase are known to mediate thrombin-induced endothelial shape change (Essler et al., 1998; Holinstat et al., 2003; Mehta et al., 2003; Mehta et al., 2001; van Nieuw Amerongen et al., 1998; Vouret-Craviari et al., 1998), we evaluated the effects of Rho kinase inhibition, as well as PKC ζ inhibition, on formation of inter-endothelial gaps. Studies were performed using cells treated with PKC ζ PSI to inhibit PKC ζ or cells pretreated with PMA for 24 hr to deplete conventional and novel PKC isoforms (Fig. 3D). We observed that depletion of conventional and novel PKCs failed to prevent inter-endothelial gap formation, whereas both the Rho kinase inhibitor (Y27632) and PKC ζ PSI prevented the response (Fig. 3D). We also determined the effects of inhibitors of PKC and RhoA signaling on the thrombin-activated permeability response in HMEC by measuring TER. The thrombin-induced decrease in TER (44 ± 4 %; n=6) was blocked by 80% in cells treated with pan-PKC inhibitors calphostin C (100 nM) and chelerythrine (10 μ M) (Fig. 3E). However, treatment with bisindolylmaleimide I (BIS; 30 nM), an inhibitor of conventional and novel PKC isoforms (27) and 4 α -PDD or PMA pretreatment of cells for 24 hr to deplete conventional and novel PKC isoforms were not able to prevent the thrombin-induced TER response. To address the specific role of PKC ζ , cells were pretreated with PKC ζ PSI as above. In this study, the thrombin-induced TER response was reduced by 85%. Consistent with results of junctional changes shown Fig. 3D, the Rho kinase inhibitor Y27632 also blocked the thrombin-induced decrease in TER (Fig. 3E). In control experiments, treatment of HMEC with myristoylated PKC θ peptide inhibitor had no effect on the thrombin-induced decrease in TER (data not shown).

PKC ζ depletion reduces the thrombin-induced increase in permeability in HMEC

To determine whether thrombin activation of PKC ζ is required for an increase in endothelial permeability, HMEC monolayers grown on gold-plated microelectrodes were transfected with PKC ζ siRNA, PKC α siRNA, or a mismatch (MM) control siRNA (75 nM in each case). Western blot analysis showed PKC ζ siRNA significantly reduced PKC ζ protein expression, as did PKC α siRNA for PKC α expression, compared to the MM control (Fig. 4A, inset). PKC α siRNA also reduced PKC ζ expression whereas PKC ζ siRNA had no effect on PKC α expression level. As shown in Figure 4B, PKC ζ siRNA and PKC ζ anti-sense both significantly reduced thrombin-induced decrease in TER as compared to the response in control cells, MM siRNA-treated cells, and PKC ζ sense oligo-treated cells (* $p < 0.05$; $n = 3-4$ /group). However, while PKC α depletion by siRNA or anti-sense tended to block thrombin-induced decrease in TER, the effect was not significantly different from that observed in the presence of MM siRNA or PKC α sense oligo. Together with the immunoblot analysis showing PKC ζ depletion by PKC α siRNA, these data support the notion that PKC ζ may play a more prominent role compared to PKC α in mediating the thrombin-induced hyper-permeability response in HMEC.

PKC ζ and RhoA mediate MLC phosphorylation in HMEC monolayers

To address the mechanism through which PKC ζ mediates thrombin-induced increase in endothelial permeability, thrombin-stimulated RhoA activation was measured using the GST-RBD pulldown assay and total RhoA protein expression was assessed by Western blotting in cells expressing dn-PKC ζ . Expression of dn-PKC ζ prevented the thrombin-induced activation of RhoA at 1 min (Fig. 5A), indicating that PKC ζ is involved in RhoA activation in response to thrombin in HMECs. We next addressed the effect of Rho kinase inhibitor Y27632 and PKC ζ PSI on thrombin-stimulated MLC phosphorylation. As shown by MLP phosphorylation assay (Fig. 5B), generation of phosphorylated MLC isoforms (the lower bands) following treatment of cells for 1 min with thrombin was inhibited by pretreatment with either Y27632 or PKC ζ PSI.

Finally, we addressed the role of PKC ζ and RhoA signaling in thrombin-induced MLC phosphorylation. When non-transfected HMEC were stimulated with thrombin for 2 to 5 min, there was an increased in anti-diphospho-MLC Ab staining over unstimulated control cells (Fig. 5C.a-c). To specifically address the roles of PKC ζ and RhoA signaling in MLC phosphorylation, HMEC were co-transfected with pEGFP and empty pcDNA3.1 vector (Fig. 5C.d), dn-PKC ζ (Fig. 5C.e), or dn-RhoA (N19) (Fig. 5C.f), and stimulated with thrombin for 2 min as in Fig. 5C.b. We observed marked reduction in phosphorylated MLC in GFP-positive cells expressing dn-PKC ζ (Fig. 5C.e) and dn-Rho (Fig. 5C.f) compared to vector control HMEC (Fig. 5C.d).

Discussion

The serine protease thrombin is a mediator of lung inflammation and acute lung injury in the setting of sepsis (Esmon, 2003; Esmon et al., 1999; Uchiba and Okajima, 1997). The immediate endothelial response to thrombin is the formation of inter-endothelial gaps, which are responsible for microvascular leakage of plasma protein (Garcia et al., 1986; Garcia et al., 1996; Lum and Malik, 1994; Moy et al., 1996; Rabiet et al., 1996). Thrombin binds to protease-activated receptor-1 (PAR-1) and cleaves it, thereby exposing a tethered ligand that auto-activates PAR-1 (Vu et al., 1991). Activation of PAR-1 causes a transient increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), which stimulates MLCK and endothelial cell retraction (Garcia et al., 1995).

Previous studies have demonstrated the important contribution of Ca^{2+} signaling in the mechanism of increased endothelial permeability (Tiruppathi et al., 2002). PAR-1 is coupled to the heterotrimeric GTP-binding protein G_q , which elicits the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), thus activating $\text{PKC}\alpha$. The increase in $[\text{Ca}^{2+}]_i$ following thrombin challenge is secondary to IP_3 -induced Ca^{2+} -store depletion and subsequent Ca^{2+} entry via store-operated calcium channels (Tiruppathi et al., 2002; Tiruppathi et al., 2003; Kwiatek et al., 2006). Ca^{2+} binds to the Ca^{2+} -binding protein, calmodulin, and together leads to activation of MLCK (Dudek and Garcia, 2001). Ca^{2+} -dependent $\text{PKC}\alpha$ activation is also required for the disassembly of adherens junctions (Garcia et al., 1996; Lum and Malik, 1994; Lynch et al., 1990; Sandoval et al., 2001), another important determinant of cell-cell junctional integrity and increased endothelial permeability (Sandoval et al., 2001).

Studies have also shown that thrombin can activate a parallel pathway regulating the dephosphorylation of MLC (Birukova et al., 2004; Essler et al., 1998). PAR-1 is coupled to the heterotrimeric G-protein $\text{G}_{12/13}$, leading to activation of the monomeric GTPase RhoA and its effector Rho kinase. The function of this pathway is to inhibit dephosphorylation of MLC by the myosin-associated phosphatase (Birukova et al., 2004; Essler et al., 1998). MLC phosphorylation results in the formation of actinomyosin contractile units that exert force on inter-endothelial junctions (Birukova et al., 2004; Dudek and Garcia, 2001; Sandoval et al., 2001), thereby creating inter-endothelial gaps (Garcia et al., 1986; Garcia et al., 1996; Lum and Malik, 1994; Moy et al., 1996; Rabiet et al., 1996).

In the present study, we investigated a pathway of RhoA GTPase activation which can control endothelial permeability without requiring an increase in intracellular Ca^{2+} . We showed in HMEC that thrombin activates $\text{PKC}\zeta$ in a Ca^{2+} -independent manner. Depletion of the Ca^{2+} -dependent conventional and novel PKC isoforms present in these cells failed to prevent the thrombin-induced decrease in TER. In addition, BIS, a PKC inhibitor which does not affect atypical PKC activity (Toullec et al., 1991), did not block thrombin-induced HMEC retraction, whereas pan-PKC inhibitors were able to abolish the effect of thrombin. However, using siRNA-mediated protein depletion, we did see a partial abrogation of the thrombin-induced decrease in TER in cells depleted of $\text{PKC}\alpha$, although not to the extent of that observed in $\text{PKC}\zeta$ depleted cells. $\text{PKC}\alpha$ siRNA also reduced the expression of $\text{PKC}\zeta$, suggesting that $\text{PKC}\alpha$ siRNA off target effects or the effect of $\text{PKC}\alpha$ expression in maintaining $\text{PKC}\zeta$ expression may be important considerations in interpreting these data.

To clarify the role of $\text{PKC}\alpha$ and $\text{PKC}\zeta$ in thrombin-induced increase in endothelial permeability, several approaches targeting $\text{PKC}\zeta$ were used to demonstrate the essential involvement of $\text{PKC}\zeta$. $\text{PKC}\zeta$ -specific pseudosubstrate inhibitor (PSI), $\text{PKC}\zeta$ antisense, and dn- $\text{PKC}\zeta$ were all used in addition to $\text{PKC}\zeta$ depletion with siRNA. These studies clearly demonstrated that inhibition of $\text{PKC}\zeta$ expression or activity blocked thrombin-induced increase in RhoA activity, MLC phosphorylation, actin stress fiber formation, and transendothelial electrical resistance (TER) in the HMEC cell line. We also assessed thrombin-induced $\text{PKC}\zeta$ activation by blotting for pT^{410} - $\text{PKC}\zeta$, since phosphorylation of Thr410 is required for $\text{PKC}\zeta$ activation (Le Good et al., 1998). pT^{410} - $\text{PKC}\zeta$ increased in response to thrombin while $\text{PKC}\zeta$ -PSI blocked this increase, indicating that thrombin stimulates $\text{PKC}\zeta$ activation. Taken together, these observations signify a key role for $\text{PKC}\zeta$ in mediating thrombin-induced increase in endothelial permeability.

Our results also demonstrated that $\text{PKC}\zeta$ was also both necessary and sufficient for activation of RhoA in response to thrombin in HMEC. Inhibitors of either $\text{PKC}\zeta$ or Rho kinase abolished thrombin-induced MLC phosphorylation, actin polymerization, and decrease in TER. Suppression of thrombin-activated $\text{PKC}\zeta$ signaling by transfection of

confluent HMEC monolayers with a dn-PKC ζ adenoviral construct prevented RhoA activation. Therefore, PKC ζ activation leads to stimulation of the RhoA pathway, which, as reported previously (Rabiet et al., 1996; van Nieuw Amerongen et al., 1998; 2002; Vouret-Craviari et al., 1998; Dudek and Garcia, 2001; Mehta et al., 2001; 2003; Holinstat et al., 2003; Birukova et al., 2004), signals endothelial cell retraction and increased endothelial permeability.

The present study raises the question whether the PKC ζ -RhoA pathway is functionally important in signaling the increase in permeability in microvessel endothelial cells *in vivo*. It is important to note that PKC ζ -dependent RhoA activation was not seen in HUVECs (Li et al., 2004). The basis of the difference between HMECs and HUVECs remains unclear, but may indicate that PKC ζ triggers thrombin-induced endothelial cell RhoA signaling in some endothelial cell types and not others.

In addition to G_{12/13}-dependent and G_q-PKC α -mediated RhoA activation, recent studies have shown that p63RhoGEF links G_{q/11}-coupled receptors to RhoA by directly interacting with GTP-bound G_{q/11} proteins (Lutz et al., 2005). However, a link between G_q and PKC ζ or PKC ζ and p63RhoGEF has not been shown. Perhaps a more plausible explanation for PKC ζ activation may be through the association of PAR-1 and G_i, and the activation of G $\beta\gamma$ signaling (Ellis et al., 1999; Vanhauwe et al., 2002). G $\beta\gamma$ is known to induce phosphoinositide-3-kinase β (PI3K β) activation leading to PKC ζ activation (Liu et al., 2003). In addition, Rac and cdc42 are also activated downstream of G $\beta\gamma$ and thus may play a role in PI3K-dependent PKC ζ activation (Etienne-Manneville and Hall, 2001; Fiory et al., 2004).

RhoA activation induced by PKC ζ may represent an important mechanism for sustaining the effects of thrombin in terms of increased endothelial permeability (Birukova et al., 2004; Essler et al., 1998; Holinstat et al., 2003; Mehta et al., 2001; Sandoval et al., 2001). Since the Ca²⁺ response was transient (peaking at 1 min and returning to baseline by 3 min), whereas retraction of endothelial cells as measured by TER peaked after 15 min and recovered in 2 hr, a possible function of the Ca²⁺-independent PKC ζ -RhoA pathway may be to prolong MLC phosphorylation and endothelial contractility. Another explanation for PKC ζ -mediated increase in endothelial permeability in the absence of a rise in intracellular Ca²⁺ could be that PKC ζ may increase the Ca²⁺-sensitivity of MLC phosphorylation by inhibiting MLC phosphatase (Gailly et al., 1997; Solaro, 2000; Somlyo and Somlyo, 2000; Somlyo, 2002). Inhibition of MLC phosphatase would thereby prolong MLC phosphorylation.

The signaling cascade depicting the role of PKC ζ in thrombin-induced increase in endothelial permeability is shown in Figure 6. The Ca²⁺-independent PKC ζ pathway identified in the present study may serve to prolong endothelial cell contractility despite return of intracellular Ca²⁺ concentration to baseline levels. Thus, PKC ζ activation may be important in promoting inflammation, and as such it represents a novel anti-inflammatory therapeutic target.

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Abbreviations

HMEC	human dermal microvessel endothelial cells
IEJs	inter-endothelial junctions
TER	transendothelial electrical resistance
MLC	myosin light chain
dn	dominant-negative
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate

4 α -PDD	4 α -phorbol 12,13-didecanoate
MLCK	myosin light chain kinase
ARDS	Acute Respiratory Distress Syndrome
GDI	guanine nucleotide dissociation inhibitor
HUVEC	human umbilical vein endothelial cells
PSI	PKC ζ pseudosubstrate peptide inhibitor
c	classical
n	novel
BIS	bisindoylmaleimide
MM	mismatch control
GST-RBD	glutathione-S-transferase-tagged rhotekin binding domain
SMM	smooth muscle myosin
NMM	non-muscle myosin
PAR-1	protease-activated receptor-1
DAG	diacylglycerol
IP ₃	inositol-1,4,5-triphosphate
PI3K β	phosphoinositide-3-kinase β

Figure 1. Pan-PKC inhibitors prevent thrombin-induced barrier dysfunction in HMEC

A: Confluent HMECs grown on gold-plated microelectrodes were stimulated with thrombin (50 nM) in the presence and absence of PKC inhibitor calphostin C (100 nM). Thrombin stimulation resulted in a transient decrease in monolayer resistance that was blocked by calphostin C. **B:** Effect of pan-PKC inhibitor on thrombin-induced actin stress fiber formation assessed by Alexa 488 phalloidin staining. Chelerythrine (10 μ M) pretreatment of HMECs for 30 min prior to stimulation with 50 nM thrombin blocked stress fiber formation. The drug independently had no effect (not shown). Results representative of 5 experiments.

Figure 2. Depletion of conventional (c) and novel (n) PKC isoforms fails to prevent thrombin-induced increase in endothelial permeability

A: HMEC pretreated with vehicle or 500 nM PMA for 24 h were lysed and 10 µg total protein was analyzed by SDS-PAGE followed by immunoblotting for PKCα, PKCδ, or PKCζ isoforms. PKCα, PKCδ, and PKCζ proteins were detectable by immunoblotting. Pretreatment with PMA for 24 hr significantly depleted PKCα and PKCδ, while PKCζ was unaffected. **B:** Thrombin and ionomycin-stimulated increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) were measured by Fura-2 ratioetric fluorescence in HMEC grown on glass coverslips. In HMEC treated with vehicle alone, thrombin increased $[\text{Ca}^{2+}]_i$ 85% above basal levels (20 cell avg.) After pretreatment with 500 nM PMA for 24 h to deplete c and nPKC isoforms,

the increase in $[Ca^{2+}]_i$ in response to thrombin was markedly reduced. Ionomycin (1 μ M, added after $[Ca^{2+}]_i$ had returned to baseline) induced similar Ca^{2+} responses in untreated and PMA-pretreated cells. Results are representative of 3 experiments. **C:** Real-time TER was used to assess thrombin-induced increase in endothelial permeability. Depletion of c and nPKC isoforms by 24 hr pretreatment (PT) with 500 nM PMA failed to prevent the thrombin-induced decrease in TER (first arrow), whereas it prevented the PMA-induced decrease in TER (second arrow). Pretreatment of HMEC with the inactive phorbol ester, 4 α -PDD for 24 hr did not affect thrombin or PMA responses. **D:** Effects of PKC inhibitors on thrombin-stimulated MLC phosphorylation. Confluent HMEC monolayers were treated for 30 min with 10 μ M chelerythrine or 24 hr with 500 nM PMA and then stimulated for 1 min with 50 nM thrombin (Thr). Myosin was detected in unstimulated cells and after 1 min thrombin treatment using an anti-MLC Ab which recognizes non-phosphorylated bands (2 top bands), monophosphorylated SMM (middle band), and diphosphorylated myosin bands (2 bottom bands). Note the appearance of diphosphorylated myosin bands following stimulation of cells with thrombin for 1 min, prevention of MLC phosphorylation by chelerythrine, and the lack of effect of PMA pretreatment of cells on MLC phosphorylation. **E:** Effect of rottlerin on MLC phosphorylation. Cells were treated with vehicle or PMA for 24 hr, and then with PKC δ inhibitor rottlerin (10 μ M) for 30 min. Thrombin (50 nM) increased MLC phosphorylation in cells treated with rottlerin alone or after PMA pretreatment. Results are representative of 3 experiments.

Figure 3. Activation of PKC ζ by thrombin in HMEC signals increased permeability

A: Confluent HMEC monolayers were immunostained with anti-PKC ζ antibody before and 5 or 15 min after thrombin treatment (50 nM). The increased cortical PKC ζ staining after thrombin treatment indicates translocation of PKC ζ from the cytosol to the membrane, which is indicative of PKC ζ activation. **B:** Western blot analysis for pT⁴¹⁰-PKC ζ was used as an indication of PKC ζ activation by thrombin as the phosphorylation of this residue is known to be required for PKC ζ activation. Densitometry results from 3 different western blots were averaged and are presented as the change in the amount of pT⁴¹⁰-PKC ζ detected after stimulation with thrombin. Thrombin induced an increase in PKC ζ phosphorylation that peaked 15 after stimulation. **C:** HMECs were pretreated with buffer alone or 10 μ M

chelerythrine for 30 min, stimulated with thrombin (50 nM) for 1 or 5 min, lysed, and separated by SDS-PAGE on 10% gels. Western blots showed increased PKC ζ phosphorylation after thrombin in control cells, which was abolished in cells pretreated with chelerythrine. **Inset:** HMECs were pretreated with buffer alone or 20 μ M PKC ζ -PSI for 30 min, and then stimulated with thrombin for 1 min. Immunoblot analysis of pT⁴¹⁰-PKC ζ levels in 20 μ g whole cell lysates indicated PKC ζ was activated after 1 min and that this response was prevented by PKC ζ -PSI. Both western blots exhibited specificity of T⁴¹⁰ - PKC ζ phosphorylation in response to thrombin. **D:** Phalloidin staining was used to monitor inter-endothelial gap formation in untreated (control) and thrombin-treated monolayers (5 min). Untreated, 24 hr PMA-pretreated (500 nM), Y27632-treated (500 nM), or PKC ζ -PSI (20 μ M)-treated HMEC monolayers were compared. Note that prolonged treatment of cells with PMA to deplete conventional and novel PKC isoforms did not block gap formation, whereas Rho kinase (Y27632) and PKC ζ inhibitors (PKC ζ -PSI) prevented the response. Results representative of 3 experiments. **E:** HMECs grown on gold-plated microelectrodes were pretreated with inhibitors for 30 min and then challenged with 50 nM thrombin. The maximum decrease in TER was normalized across experiments with the response to thrombin in untreated cells equal to 100%. Data are expressed as percent change in TER relative to control. Pretreatment of cells for 24 hr with 500 nM PMA or 4 α -PDD, or with 30 nM BIS did not block thrombin-induced decrease in TER. Chelerythrine (Chel; 10 μ M), Calphostin C (CalC; 100 nM), PKC ζ pseudosubstrate peptide inhibitor (ζ PSI; 20 μ M), or Rho kinase inhibitor Y27632 (500 nM) reduced (*p <0.05) the thrombin response. Mean $\pm$ SEM, n = 3-5.

Figure 4. PKC ζ depletion reduces thrombin-induced increase in permeability in HMEC
A: HMEC at 80% confluence were transfected with PKC ζ siRNA, PKC α siRNA or mismatched (MM) control siRNA (2.75 μ g/ml) using Dharmafect-1 (5 μ l/ml). 24 hr post-transfection, cells were seeded on gold-plated microelectrodes and grown to confluence (48 hours). Cells were then serum-starved for 2 hr and treated with thrombin (50 nM) and the resulting change in TER was measured. PKC α and PKC ζ reduced the thrombin-induced decrease in TER compared with cells transfected with MM control siRNA or mock-transfected (control) cells. **Inset:** Western blot showing reduced PKC α expression in cells transfected with PKC α siRNA and reduced PKC ζ expression in cells transfected with PKC ζ . Expression of PKC α and PKC ζ remained unaffected in cells transfected with MM control

siRNA. PKC α siRNA depleted PKC α protein expression by more than 95% but also resulted in significantly reduced PKC ζ expression. PKC ζ siRNA depleted PKC ζ by roughly 60% but did not affect PKC α expression (n=3). GAPDH was used as a loading control. **B:** Bar graph represents mean data (n=3-4) reflected in A. Thrombin-induced decrease in TER was significantly reduced by PKC ζ siRNA compared to MM siRNA, and by PKC ζ anti-sense (AS) relative to PKC ζ sense (S) oligo (see Methods for details)(* = p<0.05). The responses in cells treated with PKC α AS and siRNA tended to be lower but were not statistically different from sense and MM controls.

Figure 5. PKC ζ and RhoA-mediated MLC phosphorylation in HMEC monolayers

A: Confluent HMEC monolayers were treated for 48 hr with vehicle (control), β -galactosidase adenoviral vector (β -gal Adv), or dn-PKC ζ Adv and assayed for thrombin-stimulated RhoA activation by rotekin-binding domain pull-down assay (RBD-GST beads) and RhoA Western blot analysis. Thrombin was applied for 0 or 1 min at 37°C and cells were rinsed, lysed, and exposed to RBD-GST beads at 4°C. RhoA immunoblot assays were performed on both the beads as well as 50 μ l of whole cell lysate before exposure to the RBD-GST beads. Note the absence of active RhoA pull-down (top blot, lanes 5 and 6) in cells infected with dn-PKC ζ Adv, indicating the requirement of PKC ζ in thrombin-induced RhoA activation. **B.** Rho kinase inhibitor Y27632 and PKC ζ -PSI were tested for their ability

to block thrombin-stimulated MLC phosphorylation. Thrombin stimulation of vehicle-treated cells resulted in generation of phosphorylated myosin isoforms (lower bands). MLC phosphorylation was blocked by Rho kinase and PKC ζ inhibitors. **C:** HMEC grown on glass coverslips were stimulated with (a) vehicle or 50 nM thrombin for (b) 2 or (c) 5 min, fixed and stained with anti-diphospho-MLC Ab and DAPI, and imaged by confocal microscopy. Note the near maximal increase in MLC phosphorylation following 2 min stimulation with thrombin. To address the importance of PKC ζ and Rho signaling on MLC phosphorylation, cells co-transfected with GFP and (d) empty pcDNA3 vector, (e) dn-PKC ζ , or (f) dn-Rho were stimulated for 2 min with 50 nM thrombin as in (b). Note the absence of phosphorylated MLC in GFP-positive cells expressing dn-PKC ζ or dn-Rho. Results are representative of >10 GFP positive cells in each group from 3 separate experiments.

Figure 6. Model of Ca²⁺-independent myosin light chain phosphorylation and increased endothelial permeability

Phosphorylation of MLC (MLC₂₀) by Ca²⁺-calmodulin dependent-MLCK leads to endothelial cell contraction, whereas myosin phosphatase-induced dephosphorylation of MLC₂₀ mediates endothelial cell relaxation. Our data suggest that thrombin-activation of PKC ζ via Ca²⁺-independent mechanism leads to Rho kinase activation which may promote MLC phosphorylation by inhibiting myosin-associated phosphatase activity.