ORIGINAL ARTICLE

Angiopoietin-2 mediates thrombin-induced monocyte adhesion and endothelial permeability

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To cite this article: Rathnakumar K, Savant S, Giri H, Ghosh A, Fisslthaler B, Fleming I, Ram U, Bera A K, Augustin H G, Dixit M. Angiopoietin-2 mediates thrombin-induced monocyte adhesion and endothelial permeability. *J Thromb Haemost* 2016; **14**:1655–67.

Essentials

- Mechanism of thrombin–induced inflammation is not fully understood.
- Thrombin induced monocyte adhesion and barrier loss require Angiopoietin-2 (Ang-2).
- Ang-2 mediates vessel leakage and monocyte adhesion through SHP-2/p38MAPK pathway.
- Calcium dependent SHP2/p38MAPK activation regulates Ang-2 expression through a feedback loop.

Summary. *Background:* Thrombin imparts an inflammatory phenotype to the endothelium by promoting increased monocyte adhesion and vascular permeability. However, the molecular players that govern these events are incompletely understood. *Objective:* The aim of this study was to determine whether Angiopoietin-2 (Ang-2) has a role, if any, in regulating inflammatory signals initiated by thrombin. *Methods:* Assessment of vascular leakage by Miles assay was performed by intra-dermal injection on the foot paw. Surface levels of intercellular adhesion molecule-1 (ICAM-1) were determined by flow cytometry. Overexpression, knockdown and phosphorylation of proteins were determined by Western blotting. *Results:* In time-course experiments, thrombin-stimulated Ang-2 up-regulation, peaked prior to the expression of adhesion molecule ICAM-1 in human umbilical vein-derived endothelial cells (HUVECs). Knockdown of Ang-2 blocked both thrombin-induced monocyte adhesion and ICAM-1 expression. In addition, Ang-$2^{-/-}$ mice displayed defective vascular leakage when treated with thrombin. Introducing Ang-2 protein in Ang-$2^{-/-}$ mice failed to recover a wild-type phenotype. Mechanistically, Ang-2 appears to regulate the thrombin-activated calcium spike that is required for tyrosine phosphatase SHP2 and p38 MAPK activation. Further, down-regulation of SHP2 attenuated both thrombin-induced Ang-2 expression and monocyte adhesion. Down-regulation of the adaptor protein Gab1, a co-activator of SHP2, as well as overexpression of the Gab1 mutant incapable of interacting with SHP2 (YFGab1), inhibited thrombin-mediated effects, including downstream activation of p38 MAPK, which in turn was required for Ang-2 expression. *Conclusions:* The data establish an essential role of the Gab1/SHP2/p38MAPK signaling pathway and Ang-2 in regulating thrombin-induced monocyte adhesion and vascular leakage.

Keywords: thrombin; angiopoietin-2; inflammation; protein tyrosine phosphatase, non-receptor type 11; GAB1 protein.

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Received 25 August 2015
Manuscript handled by: W. Ruf
Final decision: P. H. Reitsma, 4 May 2016

Introduction

Endothelial inflammation and loss of its barrier properties are common pathological features of multiple diseases, such as hypertension, coronary artery disease and sepsis-induced multiple organ failure. Thrombin, a coagulatory serine protease, links vascular inflammation with coagulation, because in addition to clot formation, it imparts a pro-inflammatory phenotype through generation of inflammatory mediators (IL-6, TNF-α and MCP-1) and recruitment of immune cells to the endothelium due to

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increased adhesion molecule expression [1]. Additionally, it increases permeability by modulating the organization of cell–cell junctions between neighboring endothelial cells [2,3]. However, the signaling mechanism that triggers these pro-inflammatory effects of thrombin largely remains unexplored.

Angiopoietin-2 (Ang-2) belongs to a family of proteins that regulate inflammation and angiogenesis [4,5]. Although Ang-1 maintains endothelial quiescence through Tie2-mediated survival signaling, Ang-2 competes with Ang-1 and disrupts the endothelial barrier by inducing smooth muscle cell drop-out [6]. Ang-2 is up-regulated in diseases with a compromised endothelial barrier, be it respiratory distress syndrome, sepsis or atherosclerosis [7–10]. Recent work recognizes it as a prognostic biomarker for high mortality in sepsis patients [11]. In fact, mice engineered to overexpress Ang-2 in endothelial cells, develop sepsis-like hemodynamic changes [12]. Based on these observations, we hypothesize that Ang-2 may play a critical role in determining inflammatory responses mediated by thrombin. In the current study, we employ cultured human endothelial cells and Ang-2$^{-/-}$ mice to investigate the involvement of Ang-2 in thrombin-mediated barrier dysfunction, and to elucidate associated inflammatory signaling.

Materials and methods

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless specified. The antibody for Ang-2 was purchased from Thermo Fischer (Waltham, MA, USA). Antibodies against Phospho SHP2-Y^{542}, total SHP2, Phospho p38 MAPK, total p38, Myc tag, Gab1 and ICAM-1 were from Cell Signaling Technologies (Boston, MA, USA). Inhibitor of p38MAPK (SB203580) was purchased from Calbiochem (Germany).The intercellular adhesion molecule-1(ICAM-1) antibody for flow cytometry was from BD Biosciences (San Jose, CA, USA). Gab1 and SHP2 siRNA were from Dharmacon (Lafayette, CO, USA). Ang-2 siRNA was purchased from Integrated DNA Technologies (Coralville, IA, USA). For a detailed section on various methods please refer to supplementary materials.

Animal experiments

The animal study was reviewed and approved by the local regulatory committee (Bezirksregierung Karlsruhe, Germany; G-15/07) in accordance with the German Legislation on the Protection of Animals and the Guide for the Care and Use of Laboratory Animals. Ang-2-deficient mice originally generated as 129/J mice (kindly provided by RegeneronInc, Tarrytown, NY, USA) were crossbred into a C57BL/6N background as previously described [13]. All mice were housed in 12-h light–dark cycles with access to food and water *ad libitum* in the animal facility of the German Cancer Research Center Heidelberg, Germany. Male mice aged about 8–12 weeks were anesthetized by intraperitoneal injection of ketamine (87 mg kg^{-1}) (Pfizer, Berlin, Germany) and xylazine (13 mg kg^{-1}) (Bayer, Berlin, Germany). For rescue experiments, 1×10^7 pfu mL^{-1} of adenoviral particles encoding either the LacZ gene as negative control or human Ang-2 protein were injected into the tail vein of the study mice. Four days later expression of Ang-2 in serum was confirmed via ELISA and a Miles assay was performed.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) for Ang-2 in tissue culture supernatants and serum samples were quantified in duplicates using the Human Ang-2 Quantikine assay kit from R&D (North Kingstown, RI, USA), according to the manufacturer's protocol.

Miles assay

Miles assay was performed by intradermal injection on the foot paw of different doses of thrombin (0.5, 1, 5, 10 and 20 U) diluted in 20 μL phosphate buffered saline (PBS). Mice were sacrificed by cervical dislocation after 15 min; the foot pad with tissue containing extravasated dye was excised and incubated overnight in 200 μL formamide at 55 °C. Extracted dye was quantified using a plate reader set at 650 nm wavelength and values were expressed as fold increase with respect to vehicle control.

Cell culture

The protocol for isolation and culture of endothelial cells from umbilical cords was reviewed and approved by the Institutional Ethics Committee of IIT Madras as per the Indian Council of Medical Research, Government of India Guidelines. These guidelines are in accordance with the Declaration of Helsinki, which was revised in 2000. Following informed consent, endothelial cells were harvested by collagenase digestion from umbilical cords, as previously described [14]. Cells were cultured in flasks coated with fibronectin (BD Biosciences) in MCDB 131 medium with endothelial growth supplements, as previously described [15]. Cells up to the second passage were used for all experiments.

Western blotting

Cells were lysed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM PMSF and protease inhibitor cocktail from Sigma (St. Louis, MO, USA). Then 50 μg total protein was resolved on a 10% acrylamide gel and transferred to poly(vinylidene difluoride) (PVDF) membrane, followed by incubation

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with the primary antibody overnight at 4 °C. This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Blots were developed using a SuperSignal West Pico-chemiluminescent detection system (Pierce, Waltham, MA, USA). Densitometric analysis was performed using Image J version 1.45 (NIH, Bethesda, MD, USA).

Adhesion assay

Endothelial monolayers were serum starved overnight and treated with thrombin (1 U mL^{-1}), as indicated in the Results section, prior to adhesion experiments. THP-1 cells labeled with PKH-26 dye according to the manufacturer's instructions were incubated with thrombin-treated endothelial cells at 37 °C and 5% CO_2 for 3 h. Cell adhesion was assessed using an Olympus fluorescence microscope with 10× objective. Results are expressed as the number of THP-1 cells adhered to the endothelial monolayer per field of view. Each experiment was performed in triplicate and the results are summarized for a minimum of three independent experiments.

Flow cytometry

Surface staining of adhesion molecules for flow cytometry was performed as previously described [16]. Briefly, cells treated with thrombin (1 U mL^{-1}) were washed with PBS and fixed with 0.5% paraformaldehyde for 2 min at 4 °C, followed by blocking in 0.1% bovine serum albumin (BSA) for 30 min at room temperature. The cells were incubated with primary antibody for 1 h in the dark, followed by trypsinization and suspension in PBS for acquisition. Mean fluorescence intensity was calculated using Flowjo software version 7.6.5 (Tree star, Ashland, OR, USA).

Immunofluorescence

For immunofluorescence, endothelial cells fixed with 4% paraformaldehyde were permeabilized with 0.25% Triton-X-100, followed by incubation with anti-vascular endothelial cadherin antibody overnight at 4 °C. Cells were then washed with PBS (twice) and incubated with secondary anti-mouse Alexa 633 antibody. Images were captured using a Zeiss LSM 580 with 20× objective.

Ca^{2+} imaging

Cytosolic calcium ($[Ca^{2+}]_i$) was measured using ratiometric dye Fura-2. HUVECs were incubated with 10 μM Fura-2-AM for 30 min in Hanks' Balanced Salt Solution (HBSS) at room temperature and subsequently washed twice with dye-free HBSS. The coverslip containing dye-stained cells was placed in a recording chamber mounted on an Olympus IX-71 inverted microscope fitted with an Andor CCD camera (Andor Technology, Belfast, UK). The cells were continuously perfused with HBSS. Cells were irradiated with dual excitation wave lengths of 340 and 380 nm, while capturing the emission signal at 510 nm with the help of compatible excitation and emission filters (Chroma Technology, Bellows Falls, VT, USA). Rapid switching between the excitation filters was done using a Lambda DG-4 system (Sutter Instrument Company, Novato, CA, USA). Offline ratio-metric analysis was derived from background-subtracted images using Andor IQ software (Andor, Belfast, UK).

Electric cell-substrate impedance sensing

Cells were plated onto fibronectin-coated (30 μg mL^{-1}) 8W10E array (Applied Biophysics, Troy, NY, USA) at a density of 1×10^5 cells per well. The time-dependent impedance was measured at 37 °C and 5% CO_2 using an ECIS 8Z model (Applied Biophysics) at 4000 Hz. Once the impedance was stabilized, the cells were transfected with control oligo and Ang-2 siRNA and incubated for an additional 48 h, followed by serum starvation for 6 h. A change in impedance was recorded for about 1 h after adding thrombin (1 U mL^{-1}). Data are represented as normalized impedance across different experimental conditions.

siRNA transfection

Endothelial cells were transfected with 150 nmol of Gab1 and SHP2 siRNA (ON–TARGET plus siRNA mix consisting of four different siRNA sequences from Dharmacon) or with 50 nmoles of Ang-2 siRNA (IDT) using Lipofectamine 2000 from Invitrogen (New York, NY, USA) as transfection reagent, according to the manufacturer's instructions. For transient transfection, cells grown to 70–80% confluence were transfected with 2 μg of plasmids with Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions.

Adenovirus transfection

The generation of recombinant adenoviruses for WTGab1 and Y627F Gab1 has been previously described [17]. Confluent cultures of endothelial cells were infected with the respective adenovirus at an MOI of 30 in serum-free medium overnight. The following day, cells were washed extensively with PBS, and incubated with complete growth medium with 10% FBS for an additional 48 h. Overexpression of proteins was confirmed by Western blotting.

Statistics

Data are expressed as mean ± SEM and statistical evaluation was performed using Student's *t*-test or one-way ANOVA, followed by Dunnett's *post hoc* test where

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appropriate, using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Ang-2 is required for thrombin-induced monocyte adhesion in vitro

Ang-2 sensitizes endothelial cells to pro-inflammatory and leakage-promoting effects of TNF-α and histamine, respectively [13,18]. This observation suggests that vascular responsiveness towards inflammatory mediators could be modulated by Ang-2 in an autocrine fashion. To determine whether or not a temporal relationship exists between Ang-2 and adhesion molecules following thrombin stimulation, time-course experiments were performed. Ang-2 was basally expressed by endothelial cells, but its expression was clearly increased by thrombin (1 U mL^{-1}) stimulation, and it peaked at 3 h and reached baseline levels at around 12 h (Fig. 1A and C). Treatment of cells with the thrombin inhibitor hirudin (0.5 μg mL^{-1}) attenuated thrombin-induced Ang-2 expression, confirming the specificity of thrombin treatment (Fig. S1A). Thrombin also stimulated the up-regulation of ICAM-1, which was, however, slightly delayed (Fig. 1A and B). Additionally, Ang-2 promoter was activated by thrombin (Fig. S1B) as measured by dual luciferase assay and it was detected in the supernatant of thrombin-treated cells 3 h after stimulation, indicating its effects on release as well (Fig. 1D).

Fitting with the effects on ICAM-1 expression, thrombin (1 U mL^{-1}, 12 h) increased monocyte (THP-1 cells) adhesion to endothelial cells. This effect was significantly attenuated by the down-regulation of Ang-2 by siRNA (50 nmol L^{-1}; Fig. 1E and F), indicating that a link exists between Ang-2 and thrombin-induced endothelial cell activation. The knockdown of Ang-2 was confirmed via Western blotting (insert Fig. 1F) and ELISA (Fig. S1C). Ang-2 knockdown also attenuated the thrombin-induced surface expression of ICAM-1, as determined by flow cytometry (Fig. 1G and H). These data indicate that Ang-2 is an essential component of pro-inflammatory thrombin-induced signaling in endothelial cells.

Ang-2 regulates thrombin-induced vascular leakage in vitro *and* in vivo

Ang-2 promotes vascular leakage [19]; therefore, its role in modulating the effects of thrombin on barrier function were assessed *in vitro* and *in vivo*. *In vitro*, in human endothelial cells, thrombin induced endothelial leakage (Fig. 2A), an effect associated with the disorganization of VE-cadherin (Fig. 2B), a junctional protein with a key role in maintenance of vascular integrity [20]. Thus down-regulation of Ang-2 prevented both thrombin-induced VE-cadherin disorganization (Fig. 2B) and consequent permeability (Fig. 2A). To determine the physiological relevance of this finding, a dose–response curve for thrombin-induced vascular leakage was performed by studying Evans blue extravasation in the murine hind limb. Thrombin caused a significant increase in vascular leakage in wild-type mice at 10 and 20 units (Fig. S1D), and this effect was attenuated in Ang-2$^{-/-}$ mice (10 units for 15 min, Fig. 2C and D). It is worth noting that in agreement with a previous report, basal permeability was reduced in Ang-2$^{-/-}$ mice [13].

Because Ang-2 is bio-available in both the extracellular and intracellular milieu, we next sought to identify which pool of Ang-2 is of significance in this cascade. We performed rescue experiments using Ang-2 expressing adenoviral constructs in Ang-2$^{-/-}$ mice. Despite the fact that circulating extracellular levels of Ang-2 were several folds higher in mice treated with Ad Ang-2 when compared with a Lac Z control (Fig. S1E), thrombin induced vascular leakage in Ad-LacZ-treated WT mice, but not in Ad-Ang-2-treated Ang-2$^{-/-}$ mice, indicating that the intracellular pool is required to mediate the effects of thrombin. All the values plotted are normalized to the WT-LacZ group (Fig. 2E).

We further observed that treatment of thrombin-naive endothelial cells with conditioned medium of thrombin-treated cells did not enhance adherence of monocytes (Fig. 2F), indicating that a secreted pool of angiopoietin-2 is ineffective in inducing both endothelial inflammation and barrier dysfunction in response to thrombin. The levels of Ang-2 in the culture supernatant were verified by ELISA (Fig. S1F). In concurrent experiments we also inhibited Ang-2 build-up in the medium by overexpressing constitutively active eNOS, which is known to inhibit Ang-2 release [21]. Levels of Ang-2 in the culture supernatant were diminished (Fig. 2G) without affecting the total cellular protein content of Ang-2 (Fig. S1G). It was also observed that thrombin-mediated ICAM-1 induction was not affected (Fig. 2H), indicating that the extracellular, secreted Ang-2 is of minimal consequence in thrombin-induced, Ang-2-dependent endothelial inflammation.

Ang-2 mediates thrombin-induced calcium signaling

Our data show that extracellular Ang-2 is dispensable for both vascular leakage and monocyte adhesion. A previous study showed that chelation of intracellular calcium abrogated thrombin-induced endothelial permeability and blocked activation of inflammatory signaling [22,23]. Because Ang-2 knockdown blocked the early permeability effect (which occurs within minutes) as well as late inflammatory effects of thrombin (at 12 h), we hypothesized that this effect could be because of its ability to regulate the rise of intracellular calcium, a common mediator that could link the early and late effects of thrombin. As can be seen from Fig. 3(A), thrombin treatment transiently increased intracellular calcium within seconds. Intriguingly, the rise in both basal and thrombin-induced cytosolic calcium was

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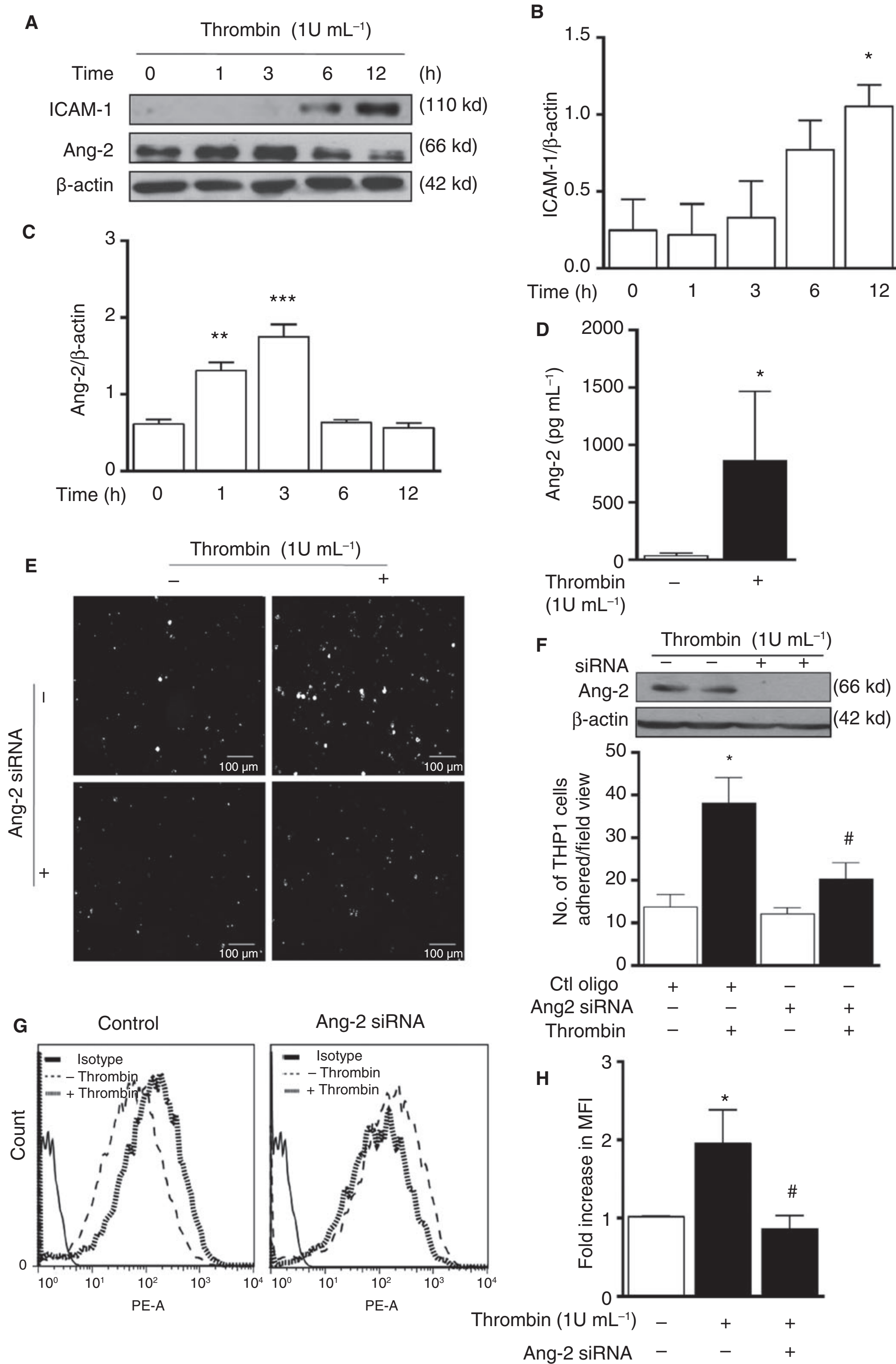

Fig. 1. Expression of Ang-2 and intercellular cell adhesion molecule-1 (ICAM-1) in response to thrombin treatment. (A) Western blot for time course of thrombin treatment (1 U mL^{-1}) on protein expression of Ang-2 and ICAM-1. (B) Bar graph summarizing data for ICAM-1 protein expression (n = 3). (C) Bar graph summarizing data for Ang-2 protein expression (n = 3). (D) Ang-2 levels in supernatants of thrombin-treated cells after 3 h. (E) Effect of Ang-2 siRNA on thrombin-induced THP-1 adhesion on human umbilical vein endothelial cells (HUVECs); magnification 10×, scale bar corresponds to 100 μm (n = 3). (F) Bar graph summarizing the result of the adhesion assay, Western blot for knockdown of endogenous Ang-2 protein by siRNA (Inset). (G) Representative histogram for surface expression of ICAM-1 in Ang-2 knockdown cells in response to thrombin (n = 3) and (H) corresponding bar graph depicting mean fluorescence intensity for ICAM-1 levels. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control and #P < 0.05 vs. thrombin.

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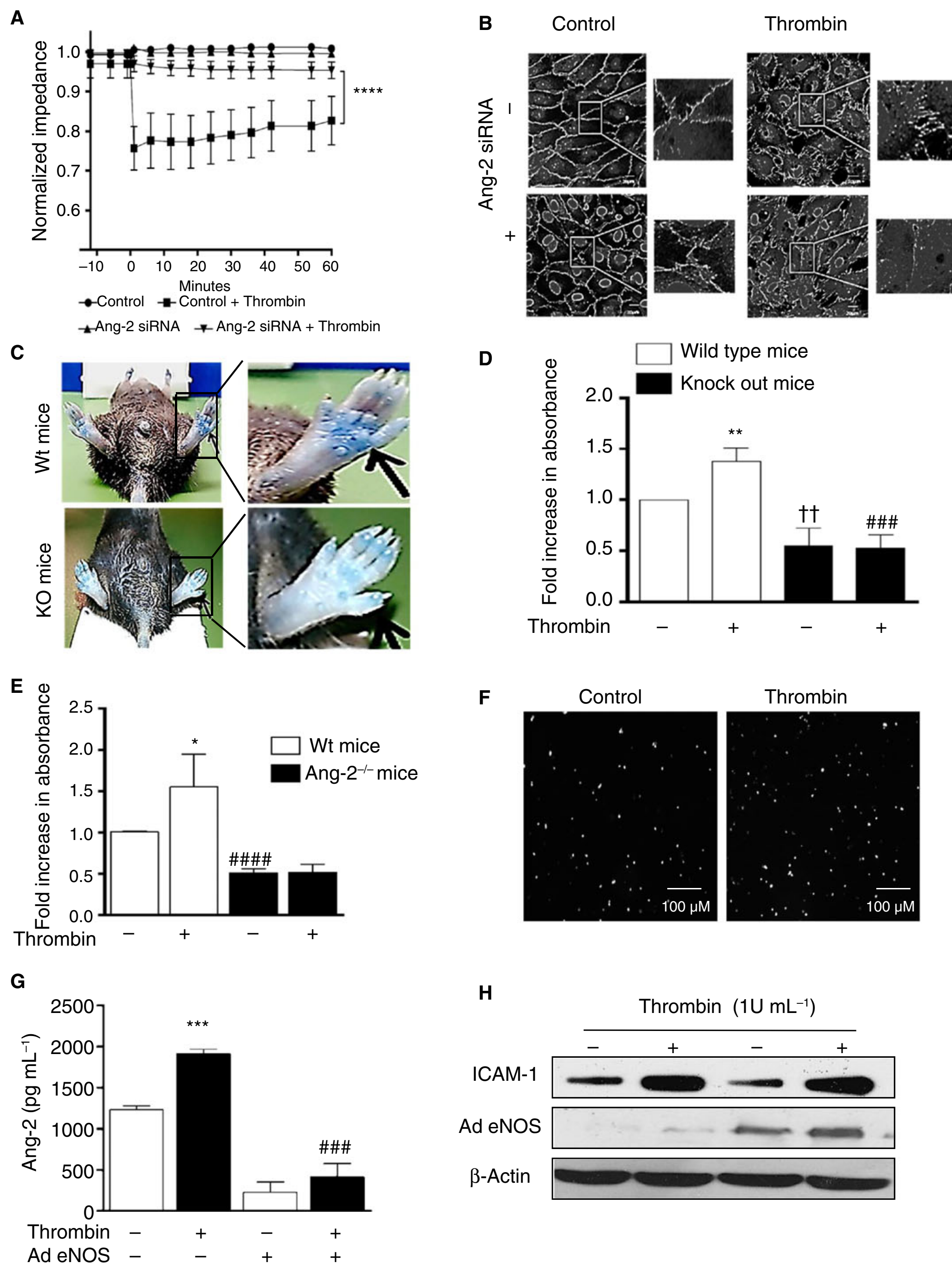

Fig. 2. Ang-2 silencing inhibits thrombin-induced vascular leakage. (A) Electrical impedance of human umbilical vein endothelial cells (HUVECs) treated with Ang-2 siRNA cultured on fibronectin-coated Electrical Cell-substrate Impedance Sensing electrode arrays stimulated with thrombin. (B) Vascular endothelial-cadherin disassembly after thrombin treatment (1 U mL^{-1} for 30 min) in control and Ang-2 knockdown cells. (C) Representative image for vascular leakage between wild-type and Ang-$2^{-/-}$ mice (n = 6). Arrows indicate extravasated dye in hind limb foot pad. (D) Bar graph comparing basal and thrombin-induced vascular leakage of Evans blue dye between wild-type and Ang-$2^{-/-}$ mice. (E) Miles assay in wild-type mice and Ang-2 overexpressing knockout mice. (F) Adhesion assay with conditioned media of control and thrombin-treated cells. (G) ELISA for Ang-2 in supernatant of cells overexpressing Ad eNOS with and without thrombin treatment. (H) Thrombin-induced intercellular cell adhesion molecule-1 (ICAM-1) expression in cells overexpressing Ad eNOS. Data are represented as mean ± SEM. $^{****}P < 0.0001$ vs. Ang-2 knockdown with thrombin treatment, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. wild-type mice phosphate buffered saline (PBS) control, $^{\dagger\dagger}P < 0.01$ vs. wild-type PBS control and $^{\#\#\#\#}P < 0.0001$, $^{\#\#\#}P < 0.001$ vs. thrombin treatment.

significantly attenuated upon down-regulation of endogenous Ang-2 via siRNA (Fig. 3A and B). To examine if there is any link between early Ang-2-dependent calcium rises and the later effect of thrombin, we chelated intracellular calcium using BAPTA/AM (20 μM) and performed flow cytometry to determine the surface levels of ICAM-1.

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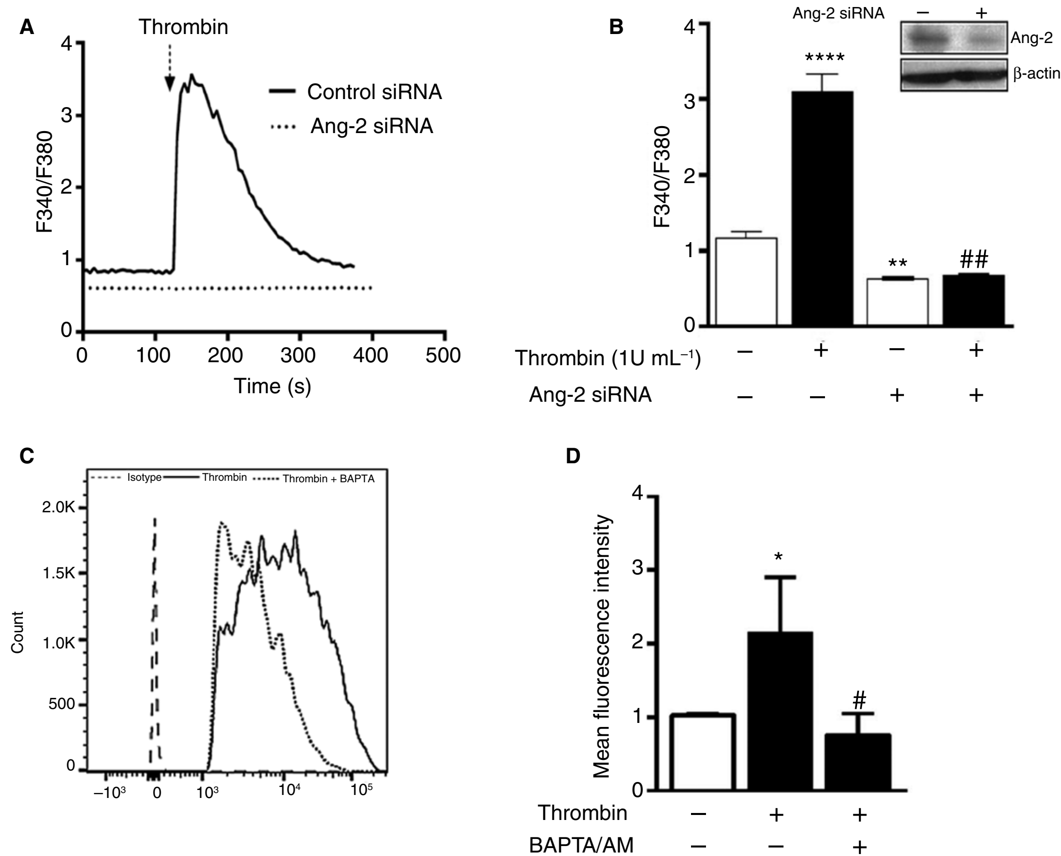

Fig. 3. Thrombin-induced endothelial calcium flux requires Ang-2. (A) Representative graph for endothelial calcium rise in thrombin-treated cells ± Ang-2 siRNA. (B) Bar graph comparing calcium spike in thrombin-treated cells ± Ang-2 siRNA. (C) Representative histogram for surface expression of intercellular cell adhesion molecule-1 (ICAM-1) in BAPTA/AM (20 μM) treated cells in response to thrombin (n = 4). (D) Corresponding bar graph depicting mean fluorescence intensity for ICAM-1 levels. Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 and ****P < 0.0001 vs. control, #P < 0.05 and ##P < 0.001 vs. thrombin.

Consistent with the data for Ang-2 knockdown (Fig. 1G and H), chelation of intracellular calcium attenuated the increase in surface ICAM-1 levels (Fig. 3C and D). These observations suggest that the Ang-2-dependent calcium signal acts as a mediator between the early and late effects of thrombin treatment.

Thrombin-induced monocyte adhesion requires SHP-2/p38MAPK activation

From the above results it is clear that intracellular calcium plays a pivotal role in the thrombin-induced inflammatory pathway; we therefore wanted to study the mechanism by which it integrates early effects with later effects of thrombin, such as monocyte adhesion. Our previous publication reported that the protein tyrosine phosphatase, non-receptor type 11 (SHP-2) dependent p38MAPK pathway is involved in regulating monocyte adhesion [15]. Hence, we asked if thrombin could activate this pathway and, if it could, does it require intracellular calcium? To this end we treated endothelial cells with thrombin for various time-points and observed that thrombin activated both SHP-2 and p38MAPK as early as within 5 min (Fig. 4A). We next examined the role of intracellular calcium by pretreating endothelial cells with the intracellular calcium chelator BAPTA/AM for 1 h (20 μM) before adding thrombin (1 U mL^{-1}). BAPTA/AM treatment completely abolished both SHP-2 and p38MAPK activation (Fig. 4B), highlighting the importance of intracellular calcium. To confirm that they belong to the same pathway, we performed a monocyte adhesion assay with p38MAPK inhibitor SB203580 (20 μM) and SHP-2 siRNA and we observed that both the treatments attenuated thrombin-induced monocyte adhesion (Fig. 4C–E). All these observations suggest that SHP-2 and p38MAPK activation is calcium dependent and are required for thrombin-induced monocyte adhesion.

Gab1/SHP-2 axis regulates thrombin-induced p38MAPK activation and Ang-2 expression

We next examined the role of the scaffold protein, Grb2 associated binder protein-1 (Gab1), as it can physically interact with SHP2 and activate MAPK [15,24]. Moreover, thrombin is reported to induce rapid tyrosine phosphorylation of Gab1, which can physically interact

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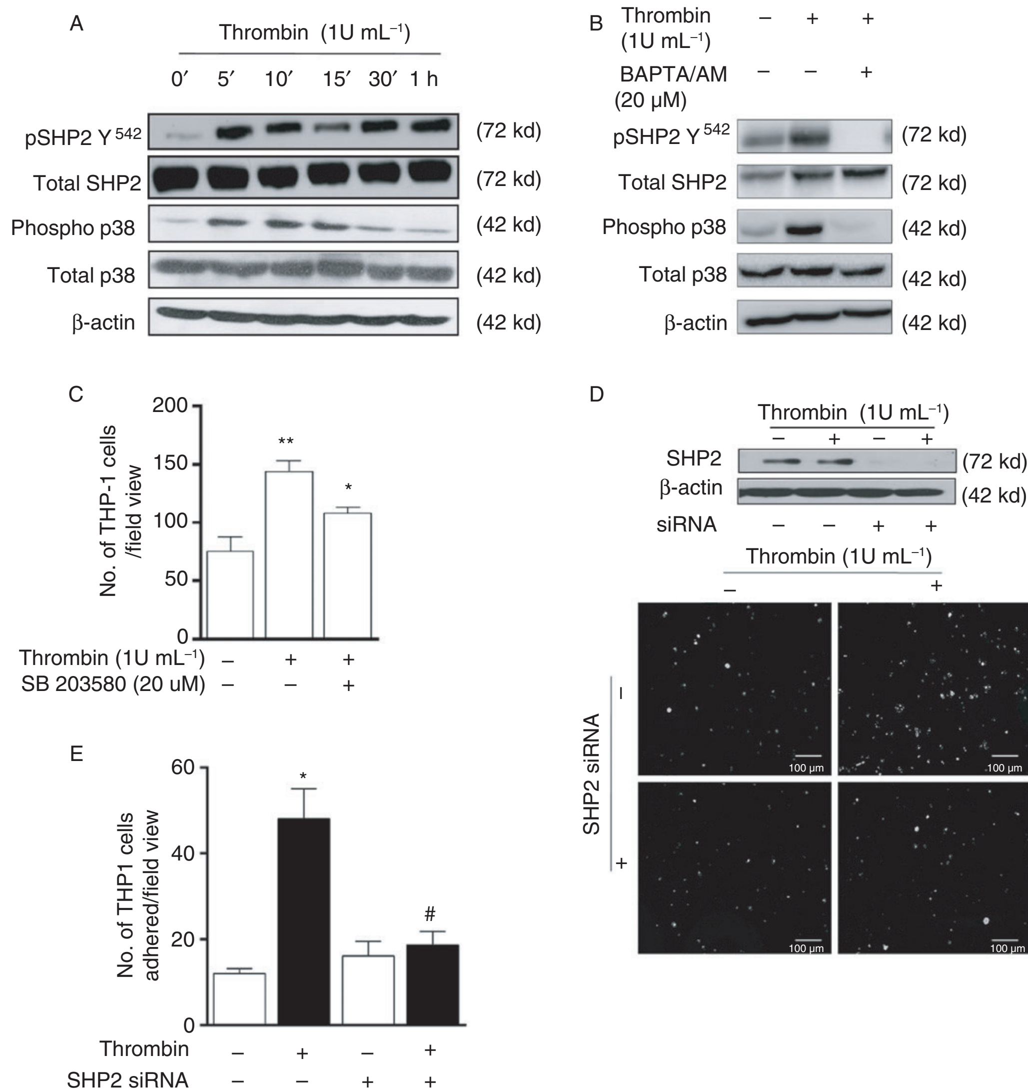

Fig. 4. Calcium-dependent activation of p38MAPK and SHP-2 activation by thrombin regulate monocyte adhesion. (A) Time course for SHP-2 and p38 activation after thrombin challenge for various time-points. (B) SHP-2 and p38 MAPK activation by thrombin with and without BAPTA/AM pretreatment (20 μM for 1 h). (C) Bar graph depicting adhesion of THP-1 cells in response to thrombin with or without p38 inhibitor SB203580 (20 μM, $n = 3$). (D) Representative image depicting adherence of PKH26-labeled THP-1 cells on treatment with control oligo and SHP-2 siRNA in the presence and absence of thrombin. (E) Bar graph summarizing monocyte adhesion in control vs. SHP2 siRNA-treated cells. Magnification: 10× with scale bar corresponding to 100 μm ($n = 3$). Data are represented as mean ± SEM for a minimum of three experiments. $*P < 0.05$, $**P < 0.01$ represent statistical significance vs. corresponding control and $\#P < 0.05$ vs. thrombin.

with SHP2 [25]. Also, we and others have earlier identified Gab1 to be a major signaling partner for SHP2 in endothelial cells [15,24]. We therefore speculated that Gab1 might have a casual role in this thrombin-mediated cascade. The time course with thrombin treatment showed an increase in total protein levels of Gab1 around 3 h that reached baseline levels around 12 h, mirroring the effects of thrombin on Ang-2 expression (Figs 1A and 5A). We therefore asked whether overexpression of Gab1 alone could increase Ang-2 levels. Indeed, overexpression of WT Gab1 was sufficient to up-regulate Ang-2 expression and release similar to thrombin challenge (Fig. 5B and C), whereas a mutant of Gab1 generated by replacing tyrosine residue at 627 by phenylalanine (YFGab1), which cannot bind SHP2 and thus cannot activate SHP2, failed to reproduce these effects (Fig. 5B and C). From this result it is conceivable that Gab-1 and SHP-2 together might regulate Ang-2 expression by thrombin (later effect as shown in Fig. 1A) and thus promote a positive feedback loop to sustain thrombin-mediated inflammatory effects. We confirmed this hypothesis by treating human endothelial cells with siRNA directed against Gab1 and observed that both basal and thrombin-induced Ang-2 expression were significantly attenuated (Fig. 5D). To further support this hypothesis, knockdown of SHP-2 also abolished thrombin-mediated Ang-2 up-regulation (Fig. 5E). Besides being an active phosphatase, few studies have

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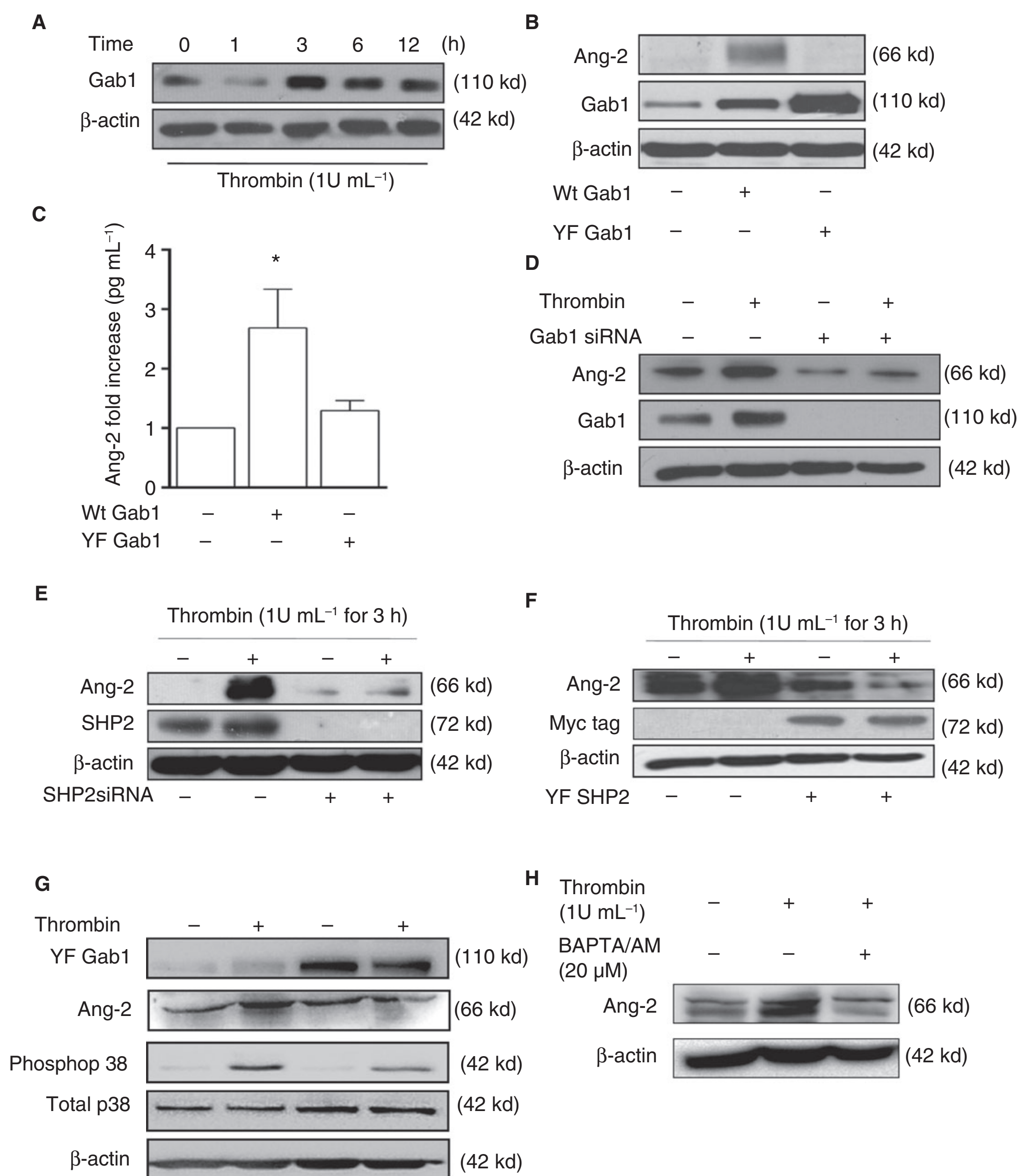

Fig. 5. Gab1/SHP2 activation regulates Ang-2 expression. (A) Time-dependent increase in Gab1 protein expression in response to thrombin (1 U mL^{-1}) (n = 3). (B) Overexpression of WTGab1 and its Y627F mutant on basal Ang-2 expression (n = 5). (C) Ang-2 levels in supernatants of cells overexpressing WTGab1 and Y627F-Gab1. (D) Representative Western blot for Ang-2 expression in cells treated with Gab1 siRNA following thrombin challenge for 3 h (n = 3). (E) Effect of SHP2 siRNA on thrombin-induced Ang-2 expression (n = 3). (F) Western blot to show the effect of YFSHP2 mutant on thrombin-induced Ang-2 expression. (G) Western blot for p38 activation (thrombin 1 U mL^{-1} for 10 min) and Ang-2 up-regulation (thrombin 1 U mL^{-1} for 3 h, n = 3) ± Y627F-Gab1 mutant. (H) Ang-2 expression induced by thrombin with and without BAPTA/AM pretreatment (20 μM for 1 h). Data are represented as mean ± SEM. *P < 0.05 vs. control.

identified an adapter-like function of SHP-2 [26,27]. We therefore wanted to check if SHP-2 enzyme activation is required for Ang-2 up-regulation. Overexpression of a non-phosphorylatable myc tagged SHP-2 mutant ($Y^{542}F$ SHP-2) repressed thrombin-stimulated Ang-2 expression (Fig. 5F), highlighting the significance of SHP-2 protein activation in this cascade.

An earlier report suggested that activation of p38MAP kinase is involved in expression of Ang-2 and its release [11,28]. Accordingly, we next examined whether the Gab1/SHP-2 axis could mediate Ang-2 expression by regulating p38 activation. Overexpression of the YFGab1 mutant diminished both thrombin-induced activation of p38MAPK and Ang-2 expression (Fig. 5G). As both SHP-2 activation and p 38MAPK activation are also calcium dependent, we next sought to determine whether chelation of calcium blocks Ang-2 expression, thus completing the feedback loop. BAPTA/AM pretreatment suppressed thrombin-induced Ang-2 expression (Fig. 5H). All these data together show that calcium-dependent

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SHP2 activation is required for thrombin-mediated p38 activation and Ang-2 expression.

Gab1 regulates thrombin-mediated monocyte adhesion

To further verify that Gab1 is indeed a component of thrombin-induced later signaling, we treated human endothelial cells with siRNA directed against Gab1. Down-regulation of Gab-1 significantly attenuated thrombin-induced expression of endothelial cell ICAM-1, as well as monocyte adherence (Fig. 6A and B). These data imply that Gab1, an upstream adapter molecule of the SHP-2 axis, promotes thrombin-induced monocyte adhesion.

Discussion

The dual roles of thrombin as a physiological regulator of hemostasis and regulator of inflammation establish a

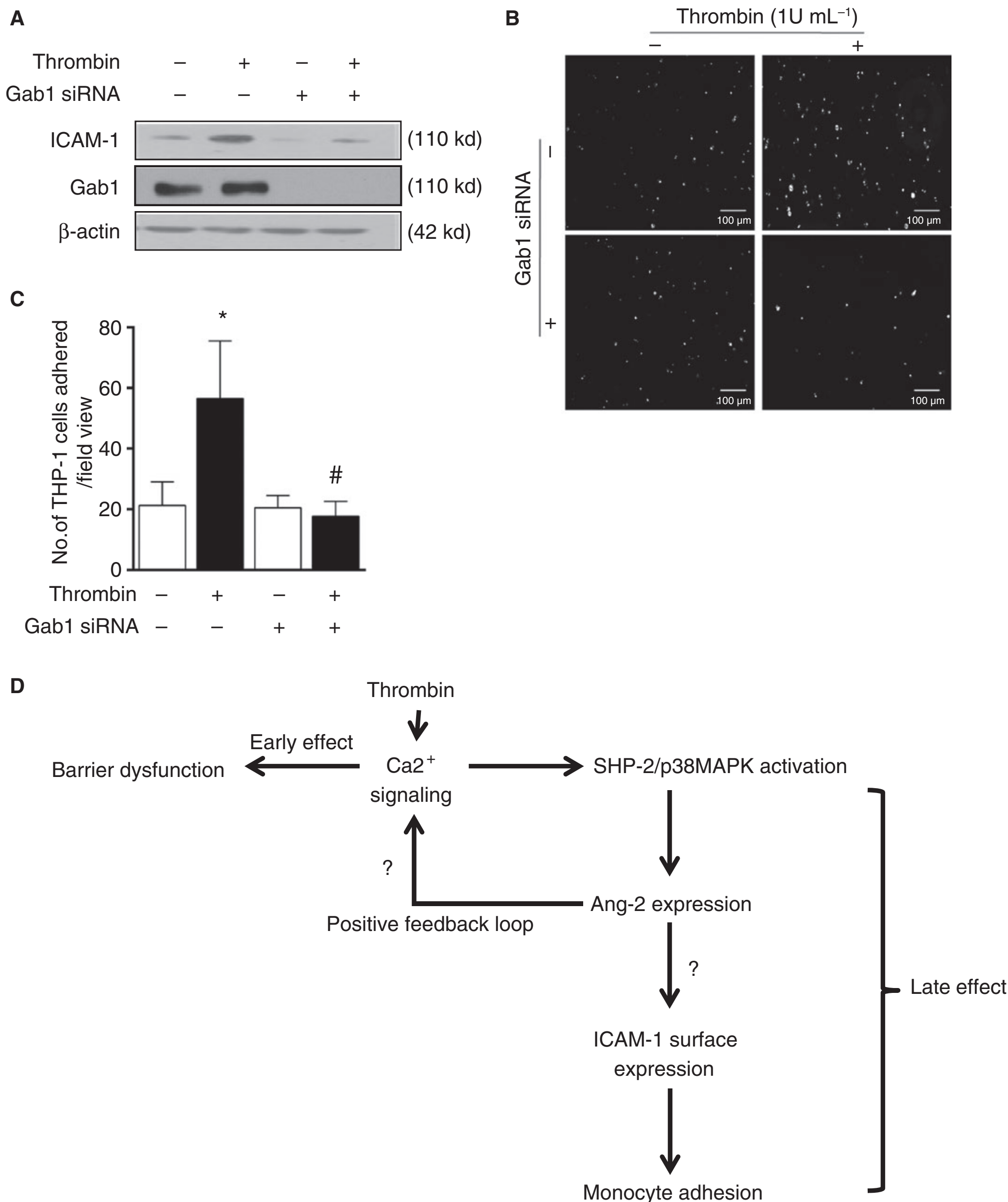

Fig. 6. Gab1 regulates thrombin-mediated monocyte adhesion. (A) Western blot for ICAM-1 levels with or without Gab1 siRNA in response to thrombin (1 U mL^{-1}) treatment. (B) Adhesion of THP-1 cells in response to thrombin with or without Gab1 siRNA. (C) Bar graph summarizing monocyte adhesion in control vs. Gab1 siRNA-treated cells. Magnification: 10× with scale bar corresponding to 100 μm ($n = 3$). (D) Summary figure depicting involvement of Ang-2 in mediating thrombin-induced early and late endothelial responses via calcium signaling. The Gab1/SHP2/p38MAPK axis is predominantly involved in thrombin-mediated later inflammatory signaling via Ang-2 up-regulation through a positive feedback loop. Data are represented as mean ± SEM. *$P < 0.05$ vs. control, #$P < 0.05$ vs. thrombin.

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functional coagulation–inflammation axis [29]. Although the cytokine storm induced during sepsis aggravates the clotting system to generate thrombin, it is also true that the thrombin thus generated further promotes inflammation [1,30]. Additionally, animal studies have positively correlated increased thrombin generation with progression of cardiovascular diseases [29]. Thus, we focused in the present study on the hitherto unknown signaling events that regulate the thrombin-driven inflammatory cascade in endothelial cells. The data of the present study demonstrate that Ang-2 is required for monocyte adhesion and vessel leakage induced by thrombin. A recent study has shown dependence on Ang-2 for ICAM-1 expression in response to TNF-α [18]. In the present study we have identified that the extracellular pool of Ang-2 is of minimal consequence in this pathway as both the rescue experiment and spent media from thrombin-treated cells failed to display vessel leakage and monocyte adhesion, respectively. Similarly, overexpression of eNOS, which prevents Ang-2 exocytosis, failed to block thrombin-induced ICAM-1 expression. Thus, it appears that intracellular Ang-2 is a primary instigator of thrombin-mediated early and late responses involving barrier dysfunction and monocyte adhesion on endothelial cells. Although Ang-2 is reported to promote tethering of the β2-integrin of monocytes to its cognate receptor ICAM-1 on the surface of endothelial cells by an unknown mechanism [4], it is conceivable that extracellular Ang-2 might continue to aggravate inflammation in the presence of other inflammatory stimuli.

Thrombin induced permeability changes and junction disorganization was attenuated in Ang-$2^{-/-}$mice and Ang-2 knocked-down endothelial cells, respectively. Thrombin-mediated alterations in endothelial cell permeability require a rise in cytosolic calcium levels and downstream activation of signaling proteins through phosphorylation [3,31]. In general, inflammatory mediators trigger endothelial permeability by increasing intracellular Ca^{2+} concentration, which activates key signaling pathways that mediate cytoskeletal reorganization (through myosin-light-chain-dependent contraction) and the disassembly of VE-cadherin at the adherents junctions [32]. Ang-2 knockdown significantly blocked the thrombin-induced intracellular calcium rise and permeability in this study. It is worth noting that histamine and VEGF-induced calcium rise is also attenuated in endothelioma cells derived from Ang-2-deficient mice [13]. The increase in $[Ca^{2+}]$i induced by inflammatory agonists such as thrombin and histamine is achieved by the generation of inositol 1,4,5-trisphosphate (IP3), activation of IP3-receptors, release of stored intracellular Ca^{2+}, and Ca^{2+} entry through plasma membrane channels. Our data with Ang-2 could possibly represent a novel link in the chain of events involved in regulating Ca^{2+} influx into endothelial cells. It is reported that increased expression of transient receptor potential cation channel, subfamily C, member 1 (TRPC1), augments Ca^{2+} influx via store-operated cation channels (SOCs) and potentiates thrombin-induced increase in permeability in human vascular endothelial cells [33]. Future investigation that aims to determine if Ang-2 is implicated in regulating calcium flux through SOCs is hence warranted.

In this study, we also have identified for the first time that the SHP-2/p38MAPK axis is acting downstream of calcium signaling in mediating later effects of thrombin, such as induction of Ang-2 expression and monocyte adhesion, through the Gab1-dependent pathway. We have shown that in the current settings, thrombin induces Ang-2 expression through this calcium-dependent SHP-2/p38MAPK pathway, thereby prolonging the inflammatory responses through a functional feedback loop (as shown in Fig. 6H). Not surprisingly, calcium-activated kinases such as PKC isoforms and Pyk2 are known to activate Gab1, resulting in Rac1 and RhoA-dependent monocyte migration in response to thrombin [34,35]. However, the kinases involved in activating SHP-2 and p38MAPK are currently not known and this requires further investigation. Thus the calcium-mediated Gab1/SHP2 axis promotes late vascular effects of thrombin in endothelial cells. To support this argument a recent report showed that SHP-2 is dispensable for the earlier permeability effect of thrombin [36]. In essence, we propose that Gab1/SHP-2/p38MAPK, apart from monocyte adhesion, regulates Ang-2 expression via a feedback loop to merely sustain the inflammatory status of the endothelium after thrombin challenge. Such feedback loops are not uncommon in human endothelial cells. Previously, we have reported that SHP2-mediated p38MAPK activation acts through a positive feedback loop to enhance the expression of arginase II, a negative regulator of the anti-inflammatory and antithrombotic molecule, nitric oxide [15]. In addition,SHP-2 might also play a critical role later in helping adhesion and subsequent transmigration of monocytes by dephosphorylating VE cadherin on tyrosine residue 731 as reported earlier [37,38].

It is worth noting that activating mutations of SHP2 (PTPN11) detected in diseases such as Noonan syndrome, acute myeloid leukemia and cervical cancer do enhance cytokine sensitivity [39,40]. Intriguingly, in a preclinical rodent model of sepsis, mitochondrial dysfunction observed in cardiac tissue was associated with enhanced mitochondrial localization of SHP2, with a concomitant decrease in tyrosine phosphorylation of mitochondrial proteins, such as porins, cyclophilin D and CytC [41]. Based on our data and the cited literature, we propose that Ang-2 takes over the central stage by promoting both permeability and monocyte adhesion by regulating calcium signals triggered by thrombin. The Gab1/SHP2 axis has a casual role in regulating the later effects of thrombin (monocyte adhesion) and Ang-2 expression, possibly to prolong the inflammatory effects of thrombin,

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as summarized in Fig. 6(C). Developing agents that rigorously inhibit Ang-2 and/or SHP2 activation could be an effective treatment modality in diseases associated with inflammatory vascular anomalies.

Addendum

K. Rathnakumar designed and performed experiments, analyzed the data and wrote the paper. S. Savant performed animal experiments. H. Giri performed adhesion experiments. A. Ghosh performed calcium imaging. B. Fisslthaler revised the manuscript for intellectual content. I. Fleming revised the manuscript for intellectual content. U. Ram revised the manuscript for intellectual content. A. Bera revised the manuscript for intellectual content and supervised calcium studies. H. Augustin supervised animal studies, analyzed data and revised the manuscript for intellectual content. M. Dixit supervised the study, designed experiments, analyzed the data, wrote the paper and revised the manuscript for intellectual content.

Acknowledgements

The authors acknowledge the expert technical assistance of A. Arul Nathan. K. Rathnakumar acknowledges the Council of Scientific and Industrial Research (CSIR), Government of India and the European Molecular Biology Organization (EMBO) for his research fellowships. Funding was provided by the Department of Science and Technology (DST) [INT/FRG/DFG/P-26/2010] and the Department of Biotechnology (DBT) [BT/PR12547/MED/30/1456/2014], Government of India; European Molecular Biology Organization (EMBO); and Deutsche Forschungsgemeinschaft (German Research Foundation) SFB834/A9.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A) Thrombin induced Ang-2 expression with and without hirudin treatment (0.5μg/ml) in HUVECs. B) Effect of thrombin treatment (1U/ml) on promoter activation of Ang-2, bar graph summarizing luciferase data after thrombin challenge in three independent experiments. C) Ang-2 protein in supernatant of endothelial cells treated with control oligo and Ang-2 siRNA. D) Bar graph summarizing dose response of thrombin-induced vascular leakage in mouse hind limb paw model of Miles Assay. E) Ang-2 protein in serum samples of mice overexpressing adenovirus coding human Ang-2. F) Ang-2 protein in culture medium of endothelial cells treated with and without thrombin (1U/ml for 12 hrs). G) Total protein levels of Ang-2 in cells over expressing constitutively active eNOS.

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