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RESEARCH PAPER

Platelet-mediated angiogenesis is independent of VEGF and fully inhibited by aspirin

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BACKGROUND AND PURPOSE

Platelets are major players in every step of vessel development through the local delivery of angiogenesis-modulating factors, including the pro-angiogenic protein VEGF and the anti-angiogenic endostatin. Although thrombin is a potent agonist and is highly elevated in angiogenesis-related diseases, studies regarding its action on the release of platelet angiogenic factors are scarce and controversial. Herein, we have investigated the role of thrombin not only in VEGF and endostatin release but also in net platelet angiogenic activity.

EXPERIMENTAL APPROACH

Human platelets were stimulated with thrombin in the presence of the various inhibitors of the signalling pathways involved in platelet activation. Supernatants/releasates were used to determine the levels of angiogenic molecules and to induce angiogenic responses.

KEY RESULTS

We found that thrombin induced the secretion of both VEGF and endostatin; however, the overall effect of the releasates was pro-angiogenic as they promoted tubule-like formation and increased the proliferation of endothelial cells. Both responses were only slightly suppressed in the presence of a VEGF receptor-neutralizing antibody. Pharmacological studies revealed that while inhibitors of PKC, p38, ERK1/2, Src kinases or PI3K/Akt exerted only partial inhibitory effects, aspirin fully blocked the pro-angiogenic activity of the releasate.

CONCLUSIONS AND IMPLICATIONS

In contrast to current belief, platelet pro-angiogenic responses are independent of VEGF and appear to be the result of the combined action of several molecules. Moreover, our data reinforce the notion that aspirin is a good candidate for a therapeutic agent to treat angiogenesis-related diseases.

Abbreviations

ENA-78, epithelial neutrophil-activating peptide; G-CSF, granulocyte-colony stimulating factor; GRO, growth-regulated oncogene; HMEC-1, Human microvascular endothelial cells 1; I-309 (CCL1), small inducible cytokine A1; MCP, macrophage chemoattractant protein; PF-4, platelet factor-4; PAR, protease activated receptor; Tie-2, tyrosine-protein kinase receptor; TPO, thrombopoietin; WPs, washed platelets

Introduction

Postnatal development of new blood vessels is mainly limited to sites of abnormal vascular surface. This process is regulated by a continuous interplay of stimulators and inhibitors of angiogenesis, and their imbalance contributes to numerous inflammatory, malignant, ischaemic and immune disorders (Carmeliet, 2005).

Platelets are being recognized as major players in every step of vessel formation (Pipili-Synetos *et al.*, 1998; Patzelt and Langer, 2012) as they are a major storage of a broad array of growth factors, chemokines, cytokines, proteases and cell adhesion molecules. The pro-angiogenic substances contained within platelets include VEGF, basic fibroblast growth factor, PDGF, EGF and stromal cell-derived factor-1α. Additionally, anti-angiogenic molecules are secreted by platelets and these include thrombospondin-1, endostatin, platelet factor-4, angiostatin, tissue inhibitor of metalloproteinases-1 and -4 and plasminogen activator inhibitor-1 (Peterson *et al.*, 2010).

It has been recently demonstrated that angiogenic factors are packed into morphologically distinct populations of alpha-granules in megakaryocytes and platelets (Sehgal and Storrie, 2007; van Nispen tot Pannerden *et al.*, 2010; Chatterjee *et al.*, 2011; Kamykowski *et al.*, 2011) and can be differentially released based on selective engagement of platelet receptors, providing a mechanism by which platelets can locally and sequentially modulate angiogenesis (Ma *et al.*, 2005; Italiano *et al.*, 2008; Bambace *et al.*, 2010; Battinelli *et al.*, 2011). In this context, intraplatelet VEGF and endostatin are the most studied pro- and anti-angiogenic factors, respectively, and they can both be differentially released upon platelet activation with different agonists such as ADP, thromboxane A_2 and protease activated receptor ligands (PARs; PAR1 and PAR4) (Ma *et al.*, 2005; Italiano *et al.*, 2008; Battinelli *et al.*, 2011). Surprisingly, although thrombin is the most potent physiological agonist known and is highly elevated in several pathological conditions where angiogenesis occurs (e.g. cancer and inflammation) (Martorell *et al.*, 2008; Han *et al.*, 2011), its action on the release of angiogenesis-modulating factors from platelet granules is still a matter of controversy.

In the present study, we aimed to further investigate the release of VEGF and endostatin mediated by thrombin, elucidate the role of VEGF in the overall angiogenic effect of releasates derived from thrombin-activated platelets as well as the effect of pharmacological inhibition of the main signalling pathways involved in platelet activation. Our results show that supernatants derived from thrombin-stimulated platelets effectively trigger the release of both VEGF and endostatin, but the net biological activity of the releasates was pro-angiogenic, as measured by an increase in endothelial cell proliferation and capillary tube formation. These angiogenic responses were independent of the action of VEGF and were mainly due to the combined action of several intraplatelet pro-angiogenic molecules. Moreover, our data showing that the pro-angiogenic activity of platelets was fully blocked by aspirin support the notion that this drug has therapeutic potential for the treatment of angiogenic-related diseases.

Methods

Preparation of human platelets

Blood samples were obtained from healthy donors who had not taken non-steroidal anti-inflammatory drugs in the 10 days before sampling. This study received the approval of the Institutional Ethics Committee and written consent from all the subjects. PRP was obtained from anticoagulated blood (sodium citrate 3.8%) by centrifugation at 180× *g* for 10 min. Platelets were washed as described previously (Etulain *et al.*, 2011), PRP was centrifuged in the presence of PGI_2 (75 nM) at 890× *g* for 10 min, washed in washing buffer (pH 6.5) at 890× *g* for 10 min, and resuspended in Tyrode's buffer at a concentration of 4×10^8 mL^{-1}. $CaCl_2$ (1 mM) was added 1 min before platelet stimulation.

Experimental design

Washed platelets (WPs) were stimulated with human α-thrombin (Enzyme research laboratories, Swansea, UK) for 5 min. Then, cells were centrifuged twice (first at 1100× *g* for 5 min and then at 9300× *g* for 5 min) in the presence of PGI_2 (75 nM), and supernatants stored at –80°C until assayed. In selected experiments, WPs were incubated for 30 min with selective inhibitors of cyclooxygenase (aspirin) (Sigma, San Diego, CA, USA), PKC (Gö-6983), p38 (SB-203580), ERK1/2 (U-0126), Src kinases (PP1), or PI-3 K/Akt (LY-294002) (Enzo Life Sciences International, Inc. San Diego, CA, USA). The drug and molecular target nomenclature conform to *BJP's Guide to Receptors and Channels* (Alexander *et al.*, 2011). The concentrations of the p38, ERK, Src, Akt, and PKC inhibitors were selected from pilot studies and were the minimal that completely suppressed phosphorylation of the specific target proteins (Supporting Information Figure S1). The concentration of aspirin or indomethacin used was the minimal that inhibited arachidonic acid but not thrombin-induced platelet aggregation (data not shown).

Immunoblotting

WPs (1×10^8 mL^{-1}) were lysed in loading buffer in the presence of a protease inhibitor cocktail (Sigma). Equivalent amounts of proteins were subjected to electrophoresis on a 12% SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After being blocked, the membranes were incubated overnight at 4°C with primary antibodies (pSrc-Tyr^{416}, pp38-Thr^{180}/Tyr^{182}, and Phospho-(Ser) PKC Substrate were from Cell Signaling, Danvers, MA, USA; pERK E-4 and pAkt1/2/3-Ser^{473} were from Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by an HRP-linked secondary antibody (Santa Cruz Biotechnology) for 1 h at 22°C. Protein bands were visualized by using the ECL reaction. Immunoblotting results were semi-quantified using Gel-Pro Analyzer 3.1 software (Media Cybernetics, Inc. Bethesda, MD, USA) and values from blot reprobes were used for normalization of data for protein loads.

Measurement of angiogenic factors by ELISA or protein array

The levels of angiogenic factors in platelet releasates were measured using ELISA commercial kits (VEGF and endostatin)

or a human angiogenesis antibody array (G series 1000 from RayBiotech, Inc. Norcross, GA, USA), according to the manufacturer's instructions.

Platelet ATP release

ATP levels were measured in a Lumi-aggregometer (Chrono-Log, Havertown, PA, USA) under stirring conditions as previously described (Etulain *et al.*, 2012). ATP was also measured under non-stirring conditions and its levels were calculated at the end of the assay by adding a known amount of ATP (Sigma, San Diego, CA, USA).

Measurement of TXB_2 release

WPs were incubated with thrombin for 5 min in an aggregometer with or without stirring at 1000 r.p.m. Addition of ice-cold PBS containing EDTA (2 mM) and aspirin (500 μM) was used to stop the reaction. The samples were centrifuged and TXB_2 was measured in the supernatants using an ELISA kit from Cayman Chemical (Ann Arbor, MI, USA) (Etulain *et al.*, 2011).

Determination of P-selectin expression

WPs were incubated with or without inhibitors, stimulated, fixed with paraformaldehyde (1%) and stained with a FITC-CD62P (anti-P-selectin) (BD Biosciences, San José, CA, USA) in PBS 0.1% FBS solution or an equivalent amount of isotype matched control antibody. Samples were analysed by flow cytometry on a FACSCalibur flow cytometer® and using CELLQUEST software (BD Biosciences, Franklin Lakes, NJ, USA) (Etulain *et al.*, 2012).

Endothelial cell culture

Human microvascular endothelial cells (HMEC-1) were obtained from the Center of Disease Control and Prevention (CDC, Atlanta, GA, USA). Cells were grown in RPMI medium supplemented with FBS (10 %), L-glutamine (2 mM), streptomycin (100 $\mu g \cdot mL^{-1}$) and penicillin (100 $U \cdot mL^{-1}$) at 37°C in a humidified 5% CO_2 incubator. In selected experiments the VEGF receptor (VEGFR) was blocked with a monoclonal neutralizing antibody against VEGFR2 (R&D Systems, Minneapolis, MN, USA).

Endothelial viability assay

Cells were analysed for changes in morphology and viability by labelling them with a mixture of fluorescent DNA binding dyes (acridine orange and ethidium bromide), as previously described (Negrotto *et al.*, 2006).

Endothelial cell proliferation

Proliferation was determined by MTT [3-(4,5-dimethylthiaxol-2-yl)-2,5-diphaenyltetrazolium bromide] assay (Promega Corporation, Madison, WI, USA). HMEC-1 cells were synchronized in Tyrode's solution with FBS (1 %) overnight, washed and incubated with platelet supernatants for 18 h before the addition of MTT solution for 3 h at 37°C. Absorbance was measured at 570 nm and the number of cells present was extrapolated from a standard curve performed for each experiment. The incubation time was determined in initial experiments and represents the population doubling time of HMEC-1 after the addition of thrombin-stimulated platelet supernatants. Positive and negative controls were Tyrode's buffer with and without recombinant VEGF (20 $ng \cdot mL^{-1}$, R&D Systems) respectively.

Capillary tube formation assay

HMEC-1 were seeded in growth factor-reduced matrigel-coated plates (Becton Dickinson Biosciences, Bedford, MA, USA) and exposed to platelet supernatants for 18 h. Tubule formation was examined under an inverted light microscope and the number of branch points in four non-overlapping fields was measured. Images were observed with a Nikon microscope, equipped with a Nikon 100X/1.4 NA objective and a 100-W mercury lamp, and were photographed using a Nikon camera and then analysed with Image J software (Arnaoutova *et al.*, 2009).

Statistical analysis

The results are expressed as the mean ± SEM and were analysed by one-way ANOVA followed by Newman–Keuls multiple comparison test to determine significant differences between groups. A *P*-value less than 0.05 was considered to be statistically significant.

Results

Thrombin induces the secretion of intraplatelet VEGF and endostatin

To clarify whether thrombin induces a differential release of VEGF and endostatin, we first measured the levels of both molecules in supernatants derived from platelets activated by different concentrations of thrombin. Figure 1 shows that thrombin was able to induce the secretion of both VEGF and endostatin in a concentration-dependent manner with a similar EC_{50} for both proteins (0.051 ± 0.005 U mL^{-1} and 0.05 ± 0.01 U mL^{-1} respectively). To evaluate whether the release of both molecules was dependent on the aggregation process, the experiments were repeated under non-stirring conditions. Figure 1A shows that the release of VEGF under stirring conditions was significantly higher than non-stirring conditions only at low concentrations of thrombin. With endostatin, no differences were observed between the conditions. It has previously been reported that activation of human platelets with ADP stimulates the release of VEGF but not endostatin, whereas TXA_2 triggers the release of endostatin but not VEGF (Battinelli *et al.*, 2011). Because thrombin-induced platelet activation results in ADP release and TXA_2 generation, to further elucidate the interaction between these agonists on the release of platelet-derived angiogenic molecules, we next determined the levels of ADP (by measuring ATP) and TXA_2 (by measuring its stable metabolite TXB_2) in the supernatants of platelets, stimulated with the EC_{50} dose of thrombin capable of inducing the release of VEGF and endostatin. The secretion of VEGF and endostatin mediated by thrombin, from stirred or non-stirred platelets, was accompanied by ATP release (Figure 1B) and TXB_2 formation (Figure 1C).

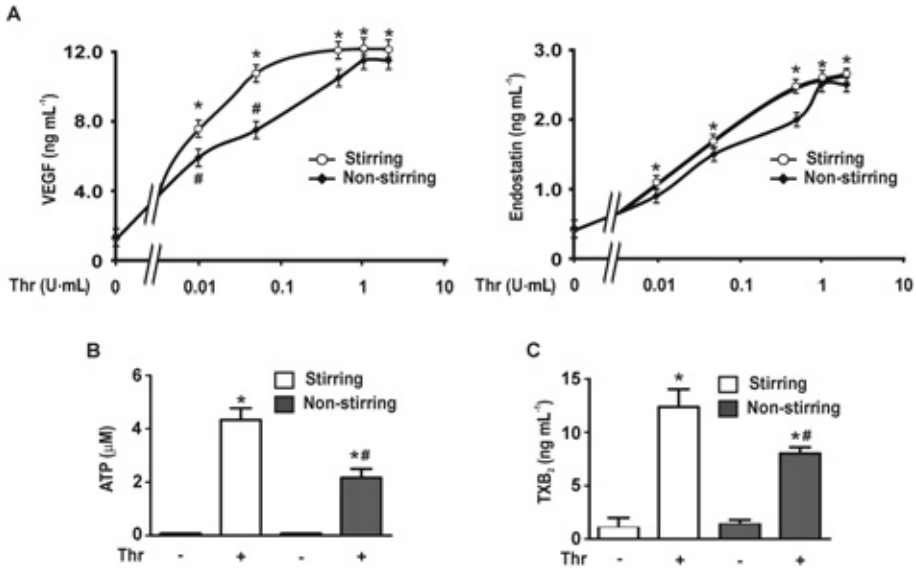

Figure 1

Thrombin induces the secretion of intraplatelet VEGF, endostatin and ATP, and TXB_2 generation. WPs were stimulated with different concentrations of thrombin (Thr) for 5 min under stirring and non-stirring conditions. (A) VEGF and endostatin levels in the supernatants were quantified by ELISA ($n = 4$). (B) and (C) WPs were stimulated with thrombin (0.05 U·mL^{-1}) for 5 min under stirring and non-stirring conditions. (B) ATP release was measured by using a lumi-aggregometer and (C) TXB_2 levels were determined by ELISA ($n = 4$, $^{*}P < 0.05$ vs. unstimulated, $^{\#}P < 0.05$ vs. Stirring).

Releasate from platelets activated with thrombin has an overall pro-angiogenic effect that is mostly a VEGF-independent response

After demonstrating that thrombin-stimulated platelets secreted both VEGF and endostatin, two factors that exert opposing effects on vessel formation, we next studied the overall angiogenic potential of platelet releasates after thrombin stimulation, on endothelial biological responses such as proliferation and capillary-like tubule formation. We found that as with VEGF, the releasates derived from platelets stimulated with low and high thrombin concentrations significantly increased both angiogenic responses (Figure 2A and B). In contrast, supernatants derived from resting platelets or thrombin in the absence of platelets showed no effect. To determine the contribution of VEGF to the overall pro-angiogenic effect of platelet releasates, we repeated these experiments in the presence of a VEGF receptor-neutralizing antibody. Surprisingly, under this condition, both endothelial proliferation and tubule formation induced by releasates from thrombin-activated platelets were only slightly suppressed (by 13–17%, Figure 2C and D). Notably, this antibody exerted a full inhibitory effect (96–98%) on angiogenic responses that were induced by recombinant VEGF (20 ng·mL^{-1}) (Figure 2C and D). These findings indicate that although intraplatelet VEGF contributes to the overall pro-angiogenic effect triggered by thrombin-activated platelet releasates, it is not essential.

Levels of angiogenic proteins in releasates of thrombin-activated platelets

Based on our findings showing that the positive regulation of angiogenesis mediated by platelet releasates is a VEGF-independent action, we next aimed to identify other angiogenic factors responsible for the observed pro-angiogenic effect. Hence, the levels of angiogenic proteins in supernatants derived from thrombin-activated platelets were compared to those from resting platelets using a human angiogenesis antibody array (absolute mean of fluorescence intensity values in Supporting Information Table S1). An analysis of the array data showed that similar to the results obtained by ELISA, VEGF and endostatin secretion were augmented in supernatants from platelets stimulated with thrombin (Supporting Information Table S1) compared to supernatants from unstimulated platelets. Additionally, some of the most elevated pro-angiogenic proteins included IL-6 and thrombopoietin (TPO) (500 to 1000-fold increase) as well as angiopoietin-1 and -2, granulocyte-colony stimulating factor (G-CSF), IL-2, GM-CSF, growth-regulated oncogene (GRO), IL-1α and β, TNFα, macrophage chemoattractant protein (MCP-3) and -4, small inducible cytokine A1 [I-309 (CCL1)], IL-8, tyrosine-protein kinase receptor (Tie-2), MMP-1 and -9, and epithelial neutrophil-activating peptide (ENA-78) (5 to 40-fold increase). Factors such as insulin-like growth factor 1, MCP-1 and EGF were moderately augmented (two to fivefold increase) whereas the levels of angiogenin, platelet-derived growth factor-BB and chemokine (C-C motif ligand 5) (also known as regulated and normal T cell expressed and secreted) were similar to unstimulated controls (Figure 3).

Signalling pathways involved in angiogenesis-mediated by platelets

To explore the intracellular signals involved in the secretion of angiogenic molecules from thrombin-stimulated platelets,

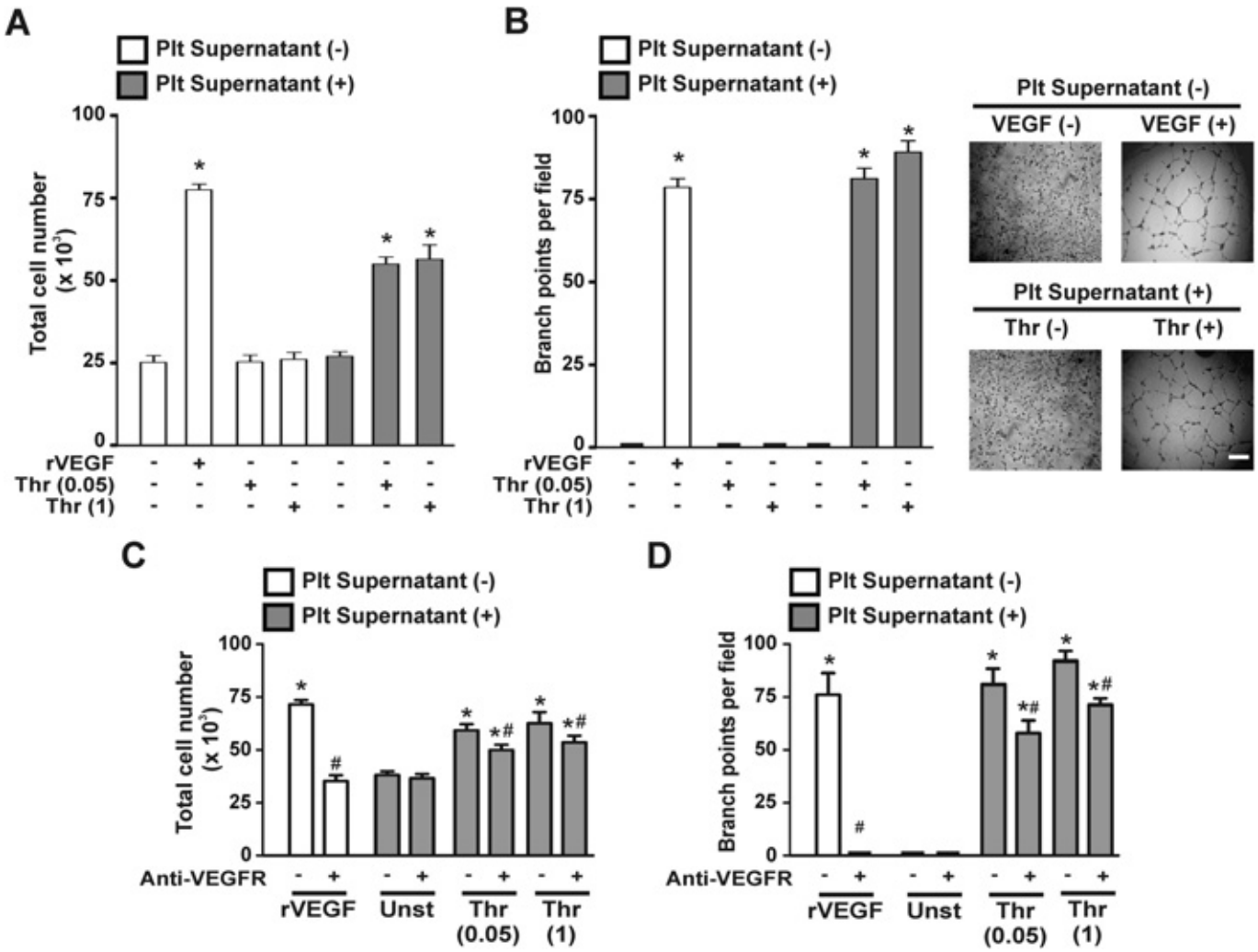

Figure 2

The releasate from platelets activated with thrombin triggered pro-angiogenic processes that are primarily a VEGF-independent response. HMEC-1 were pre-incubated in 48-well plates without (A) and (B) or with (C) and (D) anti-VEGF receptor antibody (anti-VEGFR, 2 μg·mL^{-1}) for 30 min. Cells were then stimulated with recombinant VEGF (rVEGF, 20 ng·mL^{-1}) (positive control) or with platelet (Plt) supernatants from unstimulated (Unst) or thrombin-stimulated (0.05 or 1 U·mL^{-1}) platelets after 18 h. (A) and (C) Endothelial proliferation was determined by addition of MTT reagent. The reaction was stopped and the absorbance at 570 nm was measured. (B) and (D) Tube formation in the matrigel-coated wells was analysed under an inverted light microscope, and the number of branch points in four non-overlapping fields was determined. The scale bar is 200 μm in size (n = 5, *P < 0.05 vs. unstimulated, $^{\#}P$ < 0.05 vs. without anti-VEGFR).

we next tested specific inhibitors of the major signalling pathways involved in platelet granule secretion including the cyclooxygenase-1 (aspirin), PKC (Gö-6983), p38 (SB-203580), ERK1/2 (U-0126), Src (PP1) and PI3K/Akt (LY-294002) pathways (Li *et al.*, 2010). Immunoblot studies show that all the inhibitors at the concentration employed completely blocked activation of their respective targets (Supporting Information Figure S1). As shown in Figure 4, interference with each of the main signalling pathways resulted in different degrees of inhibition of the angiogenic responses induced by platelet supernatants. For instance, endothelial cell growth and tubule formation were partially, but significantly inhibited by the blockade of PKC, p38 and ERK1/2 whereas inhibition of Src kinases or PI3K/Akt showed little or no effect respectively (Figure 4A and B). However, when platelets were activated in the presence of aspirin, both processes were completely suppressed (96% inhibition). Because aspirin can exert antiangiogenic effects in a COX-independent manner (Borthwick *et al.*, 2006) we also analysed the effect of indomethacin, a non-salicylate alternative inhibitor of COX. Like aspirin, incubation of platelets with indomethacin resulted in a complete inhibition of the platelet pro-angiogenic activity (Figure 4D and E). Moreover, treatment of platelets with aspirin did not significantly inhibit the phosphorylation of p38, ERK, Src, Akt and PKC substrates (Supporting Information Figure S1).

Although it could be argued that the inhibition of angiogenesis was due to a direct effect of these different

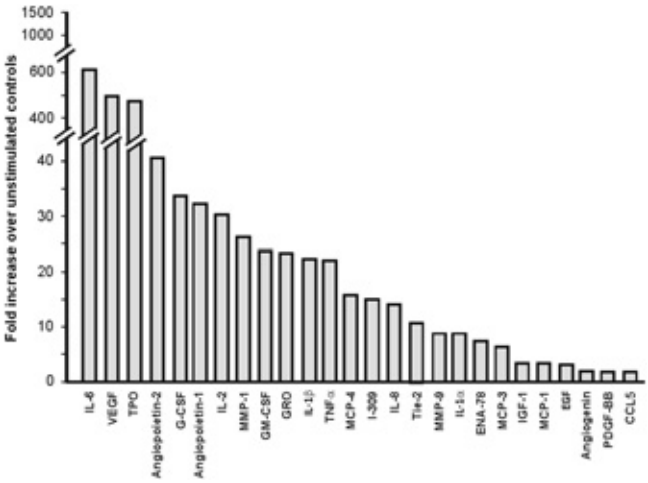

Figure 3

The levels of angiogenic proteins in the releasate of thrombin-activated platelets. WPs were stimulated with thrombin (0.05 U·mL^{-1}) for 5 min. The release of angiogenic regulators was measured using commercial array kits according to the manufacturer's instructions. The thrombin-induced release of each molecule is expressed as the fold increase over unstimulated controls (n = 2).

compounds on the endothelial cells, this hypothesis was ruled out because the addition of thrombin-stimulated platelet supernatants, which were supplemented with the inhibitors after platelet stimulation to endothelial cultures, failed to modify any of these angiogenic responses. Higher concentrations were required to inhibit these processes (Supporting Information Figure S2A and B). Moreover, taking into account that aspirin was the most powerful inhibitor and is an irreversible inhibitor of COX, we performed additional experiments using supernatants from platelets that were pre-incubated with aspirin, washed and then stimulated with thrombin. As shown in Supporting Information Figure S2C and D, platelet-mediated angiogenic processes were completely inhibited by aspirin even when the drug was removed before platelet activation.

Because the signalling pathways involved in secretion may depend on the strength of the stimulus, we next analysed whether a similar effect was observed using a higher thrombin concentration. Although the inhibitory effect of the drug on angiogenesis induced by thrombin 1 U·mL^{-1} was lower than that observed with thrombin 0.05 U·mL^{-1}, the pattern of inhibition induced by each drug was similar for both thrombin concentrations, and aspirin remained the most effective blocker of platelet-mediated angiogenesis (Figure 4A and B).

Furthermore, as the number of necrotic or apoptotic cells never exceeded 1% for all treatments, the inhibition of cell growth was not associated with a drug-mediated cytotoxic effect (Supporting Information Figure S3).

Taken together, these data demonstrate that the net pro-angiogenic effect induced by platelet-derived soluble angiogenesis modulators is regulated by distinct signalling pathways and largely depends on the action of COX-1.

Alpha granules are not all identical; they have different protein content and their release is regulated by different signalling pathways

As it has been demonstrated that not all alpha granules have the same protein content and that their release can be regulated by different signalling pathways, we aimed to identify which molecules within the platelet granules were affected by each specific inhibitor. To this end, the secretion pattern of platelet angiogenesis-modulating substances triggered by thrombin was analysed before and after blockade of the main signalling pathways. Consistent with the results observed in the endothelial responses, incubation of platelets with the different inhibitors resulted in selective blockade of the secretion of angiogenic molecules (Supporting Information Table S1). Among the 26 different pro-angiogenic molecules detected by the array, the degree to which IL-1β and IL-2, angiopoietin-1 and -2, G- and GM-CSF and TNF-α were inhibited most closely followed the inhibition pattern observed in the biological endothelial responses (Figure 5A), suggesting that these molecules are crucial mediators of the pro-angiogenic effect exerted by releasates from thrombin-activated platelets. However, as aspirin was the most effective inhibitor of the biological responses (Figure 4), we also analysed the array data to determine whether there was any molecule inhibited only by aspirin and found that the release of ENA-78 and EGF were solely but moderately (20 and 22% of inhibition, respectively) blocked by the inhibition of COX-1 (Supporting Information Table S1).

Interestingly, the array data showed that the inhibitors of PKC, p38, ERK1/2, Src and PI3K/Akt partially inhibited VEGF secretion (between 20 and 50% inhibition) by thrombin-activated platelets, whereas aspirin had almost no effect (Figure 5B). These findings were confirmed by ELISA studies (Figure 5B).

To analyse whether this differential signalling regulation also influences other non-angiogenic molecules stored either in alpha or dense granules, the expression of P-selectin and the ATP release induced by thrombin was evaluated in the presence of the different inhibitors. Figure 5C shows that as with VEGF release, inhibition of PKC activation resulted in a 50% reduction of both the expression of P-selectin and ATP secretion. However, in contrast to alpha granule release (either VEGF or P-selectin), the blockade of Src and PI3K/Akt pathways had the greatest effect on ATP release, reducing it by more than 50% (Figure 5D).

Discussion and conclusions

Platelets, in addition to maintaining haemostasis, play a critical role in regulating angiogenesis by releasing factors that promote the growth of new vessels. Although thrombin is the most powerful platelet agonist and is generated in almost all physiopathological processes involved in the formation of new vessels (Moser, 2008), the thrombin-dependent release of pro- and anti-angiogenic molecules is not clearly understood. Pioneering studies by Mohle *et al.* (1997) and Maloney *et al.* (1998) demonstrated that megakaryocytes and platelets synthesize and release VEGF upon stimulation with thrombin.

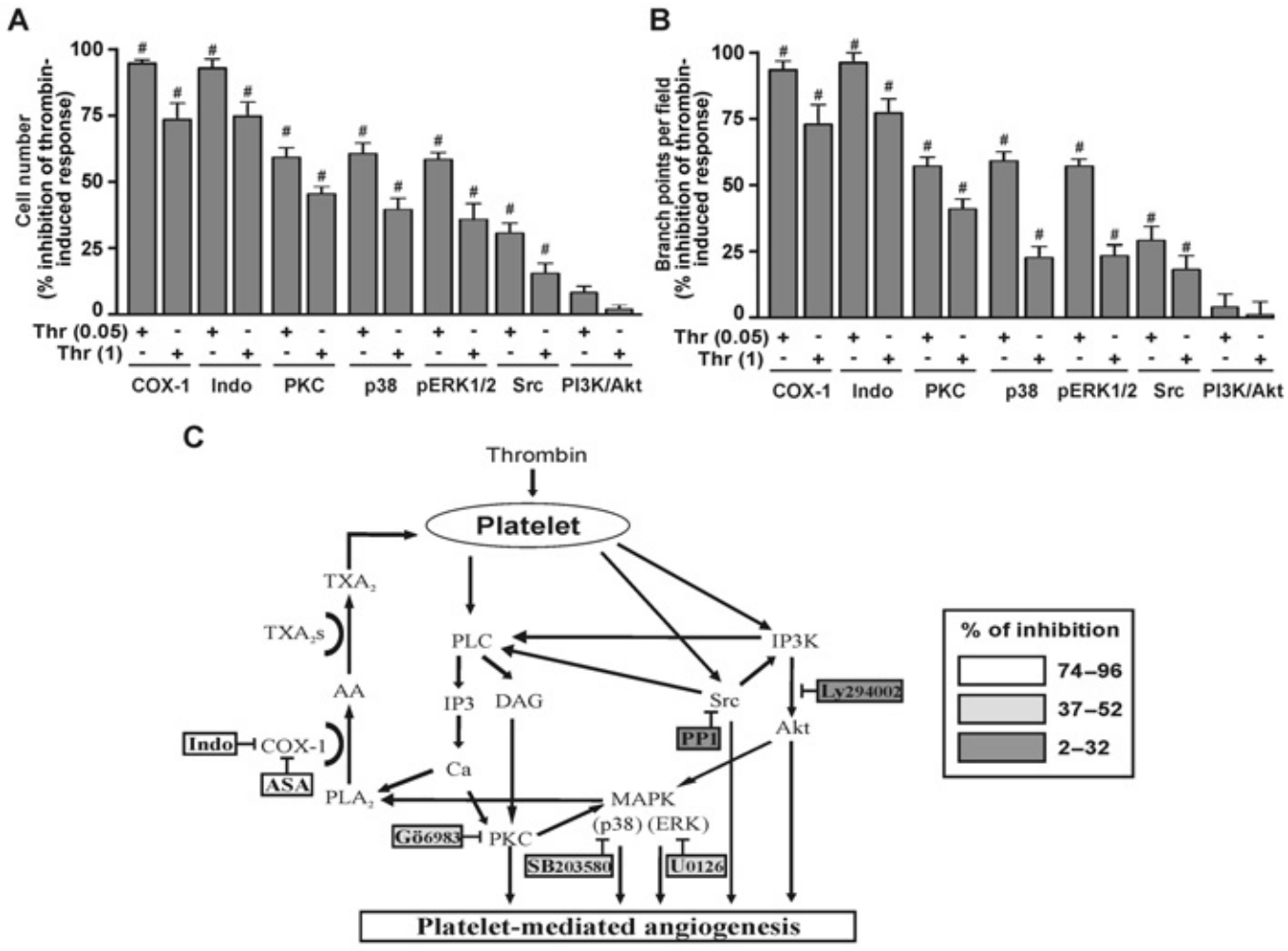

Figure 4

Signalling pathways are involved in platelet-mediated angiogenesis. WPs were incubated with or without the inhibitors of COX-1 (aspirin, 500 μM or indomethacin (Indo), 30 μM), PKC (Gö6983, 1 μM), p38 (SB203580, 25 μM), ERK1/2 (U0126, 10 μM), Src kinases (PP1, 5 μM), or PI3K/Akt (Ly-294002, 10 μM) pathways for 30 min. Platelets were then stimulated with thrombin (0.05 or 1 U·mL^{-1}) for 5 min. Platelet supernatants were obtained and used to induce angiogenic responses. (A) Endothelial proliferation was determined by the addition of MTT reagent. The reaction was stopped and the absorbance at 570 nm was measured. (B) Tube formation in the matrigel-coated wells was analysed under an inverted light microscope, and the number of branch points in four non-overlapping fields was determined. The results are expressed as a percentage inhibition of thrombin-induced control response (without inhibitor) (n = 5, $^{\#}P < 0.05$ vs. without inhibitor). (C) Schematic representation of the platelet pathways inhibited.

However, a subsequent study showed that platelet activation with thrombin triggers the secretion of neither VEGF nor endostatin because it simultaneously activates PAR-1 and -4, which counter regulate the release of both molecules (Ma *et al.*, 2005). In this study, we investigated the role of thrombin in the differential release of platelet VEGF and endostatin as well as in net platelet angiogenic activity. We found that thrombin triggered the secretion of both angiogenic molecules in a concentration-dependent manner, whereas platelet releasates promoted endothelial cell proliferation and capillary-tube-like structures in a VEGF-independent but TXA_2-dependent manner. While Maloney *et al.* (1998) demonstrated that the release of VEGF is intimately associated with platelet aggregation, our present results performed under stirring and non-stirring conditions show that secretion of either VEGF or endostatin can occur independently of the platelet aggregation response. Although stirring increased VEGF release it did not modify the secretion of endostatin. Similar results with regard to the release of VEGF, were observed by Mohle *et al.* (1997). The differential release of VEGF and endostatin was observed not only after PAR-1 and PAR-4 activation (Ma *et al.*, 2005; Italiano *et al.*, 2008), but also after activation of platelets by ADP or TXA_2 (Bambace *et al.*, 2010; Battinelli *et al.*, 2011). These findings raise the following question: what would be the net biological response when platelets are simultaneously stimulated with ADP and TXA_2? Our present data show that when both mediators are generated as a result of platelet stimulation with thrombin,

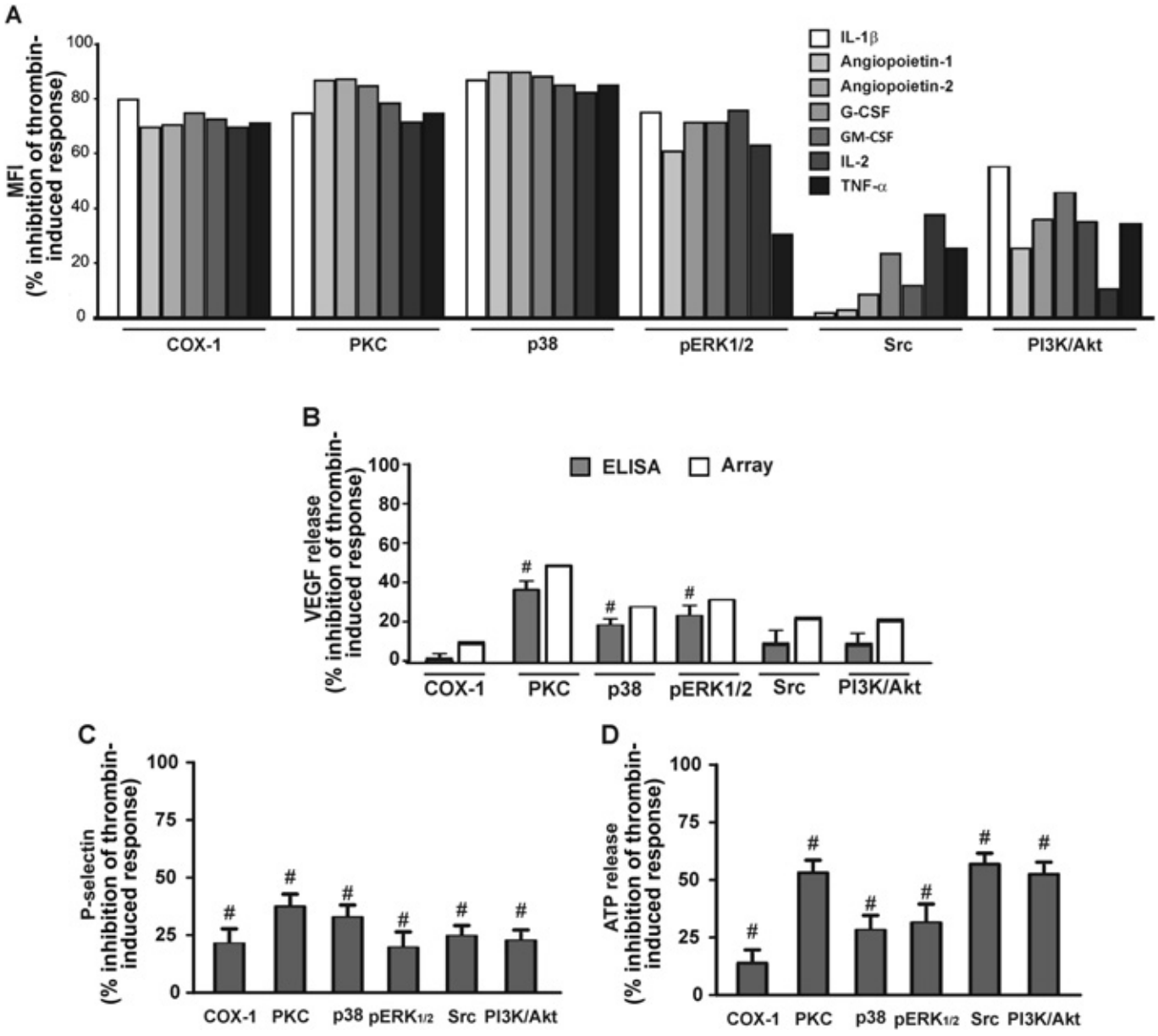

Figure 5

The signalling pathways involved in the secretion pattern of thrombin-triggered angiogenesis-modulating platelet products. WPs were incubated with or without the inhibitors of COX-1 (aspirin, 500 μM), PKC (Gö6983, 1 μM), p38 (SB203580, 25 μM), ERK1/2 (U0126, 10 μM), Src kinases (PP1, 5 μM), or PI3K/Akt (Ly-294002, 10 μM) pathways for 30 min. Platelets were then stimulated with thrombin (0.05 U·mL^{-1}) for 5 min and supernatants were obtained. The release of angiogenic regulators was measured by using commercial array kits. The graphs demonstrate (A) the mean of fluorescence intensity (MFI) of each indicated molecule, and (B) thrombin-induced VEGF release measured by ELISA (n = 3) or array (n = 2). (C) P-selectin exposure was detected by flow cytometry (n = 5) and (D) ATP release was measured by using a Lumi-aggregometer. The results are expressed as a percentage inhibition of the thrombin-induced control response (without inhibitor) (n = 5, $^{\#}P < 0.05$ vs. without inhibitor).

both VEGF and endostatin are secreted and that the net biological activity (evaluated as endothelial proliferation and capillary-tube-like structures) is distinctly pro-angiogenic.

Surprisingly and contrary to current belief, we also found that the role of VEGF in the platelet-mediated angiogenic activity appears to be negligible. Blockade of the endothelial VEGF receptor had only a weak inhibitory effect (15% inhibition) on the entire angiogenic response mediated by releasates from thrombin-stimulated platelets. It is widely recognized that in addition to VEGF and endostatin, platelet alpha granules contain several growth factors and chemokines that positively and negatively regulate angiogenesis (Peterson *et al.*, 2010). We demonstrated that after thrombin stimulation, the pro-angiogenic proteins that are elevated the most included not only VEGF but also IL-6 and TPO (500–1000-fold increase) as well as angiopoietin-1 and-2, G-CSF, IL-2, GM-CSF, GRO,

IL-1α and β, TNF-α, MCP-3 and-4, I-309, IL-8, Tie-2, MMP-1 and-9, and ENA-78 (5 to 40-fold increase).

Thus, our data indicate that the net pro-angiogenic activity is the result of the combined action of several cytokines, which was further confirmed by our pharmacological studies to elucidate the molecular signalling pathway involved in the release of these angiogenic molecules. Proliferation of endothelial cells and the formation of new vessels were completely suppressed by platelets treated with aspirin before thrombin stimulation, were partially blocked by the inhibition of the PKC, p38 and ERK pathways, and were barely affected in platelets pre-incubated with inhibitors of Src kinases and the PI3/AKT pathway. The observation that the inhibition patterns induced by each drug were similar (although less efficient in the degree of effect) when platelets were stimulated with either 0.05 or 1 $U \cdot mL^{-1}$ thrombin, indicates that regulation of platelet-mediated angiogenesis by this signalling pathway is not significantly modified by the strength of the stimulus. These results are in agreement with those of Coppinger *et al*. who demonstrated that the releasate profile observed when platelets were stimulated with high concentrations of ADP was identical to that seen with lower concentrations, although the effect of aspirin was more pronounced in platelets stimulated with the lower dose of ADP (Coppinger *et al*., 2007). Furthermore, an analysis of the individual angiogenic molecules revealed that the inhibition of IL-1β and IL-2, angiopoietin-1 and -2, G- and GM-CSF and TNF-α release by the different compounds correlated with a similar inhibition pattern observed in the proliferation of endothelial cells and tubule-like formation. Nevertheless, considering that aspirin was the most effective inhibitor of the biological responses (>95% Figure 4) it is conceivable that other molecules not represented on the array-based screening experiments might be involved.

The intriguing observation that the inhibition of specific signalling pathways of platelet activation results in the release of different pro-angiogenic molecules could be explained by the new theories of platelet granule secretion. While some recognize the existence of alpha granule subtypes with a different morphology and protein cargo that can be differentially released in response to a specific agonist (Italiano *et al*., 2008; van Nispen tot Pannerden *et al*., 2010; Chatterjee *et al*., 2011), recent studies have shown that alpha granule proteins are stochastically packaged into subdomains within single granules, and that the proteins displayed little, if any, pattern of functional co-clustering (Kamykowski *et al*., 2011). Platelet secretion, rather than having a limited thematic response to specific agonists, appears to be a stochastic process potentially controlled by several factors, such as cargo solubility, granule shape and/or granule-plasma membrane fusion routes (Jonnalagadda *et al*., 2012). Although the structure and dynamics of alpha granule secretion appear somewhat controversial and still remain to be clarified, the different theories seem to converge in the notion that selective signalling pathways might be involved in the regulation of the exocytosis of alpha granule contents.

Our findings showed that cell proliferation and tubule formation are similarly affected by the different inhibitors. This is somewhat surprising considering that the process of tubule formation on a basement membrane matrix is mainly supported by migration and differentiation, but not by proliferation of endothelial cells. Nevertheless, similar to our results, several studies have demonstrated a similar inhibition pattern of tubule formation and proliferation mediated by several pharmacological inhibitors, suggesting that the intracellular pathways governing the tubule formation and proliferation are not so distinctly separate and some may even overlap (Wang *et al*., 2005; Yu *et al*., 2006; Kim *et al*., 2008). Still, we cannot rule out the possibility that the unifying elements driving the effect of our compounds on the endothelial cell responses are lipids products of COX, even TXA_2, or platelet microparticles rather than a specific alpha granule derived protein.

With regard to VEGF, although numerous studies have suggested that this molecule plays a major role in platelet-mediated angiogenesis, we observed that selective blockade of this growth factor had almost no effect. Moreover, our data showed that aspirin failed to inhibit VEGF release but fully blocked endothelial proliferation and tubule formation. Overall, our study introduces the novel concept that VEGF is not essential for the pro-angiogenic activity of platelets, and reinforces the notion that there are multiple, redundant pathways mediating this process.

Although angiogenesis is a crucial component of the wound healing process, it also occurs in several diseases including cancer. The tumour milieu is enriched in angiogenic factors derived from the tumour, endothelial and stromal cells, or myeloid populations of tumour-associated macrophages or neutrophils recruited to the site (Weis and Cheresh, 2011; Albini *et al*., 2012). However, several *in vitro* and *in vivo* studies in animals and patients have highlighted that platelets are another relevant source of angiogenic factors that contribute to tumour progression (Ho-Tin-Noe *et al*., 2008; Pietramaggiori *et al*., 2008; Klement *et al*., 2009; Sabrkhany *et al*., 2011; Cho *et al*., 2012; Peterson *et al*., 2012). Therefore, pharmacological inhibition of platelet pro-angiogenic activity has been considered as a potential adjuvant therapy for cancer (Bambace and Holmes, 2011; Radziwon-Balicka *et al*., 2012). In this context, aspirin could be a good candidate as our present data show that it completely suppresses the angiogenic responses induced by releasates from thrombin-activated platelets. Interestingly, a recent analysis of 43 randomized trials of daily aspirin administration for the primary and secondary prevention of vascular diseases showed a significant, 12%, reduction in the risk of cancer death suggesting that inhibition of platelet activation may mediate both the cardioprotective and cancer-preventive effects of low doses of aspirin (Rothwell *et al*., 2012; Thun *et al*., 2012). Moreover, the inhibition of several pro-angiogenic molecules might overcome the drawbacks of the current single-molecule blockade, which includes the fact that tumours may circumvent the inhibition of a single angiogenic protein by alternative expression of another angiogenic factor (Sennino and McDonald, 2012).

In conclusion, we demonstrated that although thrombin is capable of stimulating the secretion of both VEGF and endostatin, the effect of releasates from thrombin-stimulated platelets is clearly pro-angiogenic. This response is independent of VEGF activity and appears to be mainly due to the combined action of several intraplatelet pro-angiogenic molecules.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Inhibition of platelet signalling pathways. WPs were incubated for 30 min with or without the inhibitors of COX-1 (aspirin, ASA 500 μM), p38 (SB203580, '++':25 μM; '+':12.5 2M), ERK1/2 (U0126, '++':10 μM, '+':5 μM), Src kinases (PP1, '++':5 μM, '+':2.5 μM), PI3K/Akt (Ly-294002, '++':10 μM, '+':5 μM), PKC (Gö6983, '++':1 μM, '+':0.1 μM) and then stimulated with thrombin (0.05 U·mL^{-1}) for 5 min. (A) Samples were immunoblotted with anti-pp38, pERK1/2, pSrc or pAKT antibodies (n = 3, $^{*}P$ < 0.05 vs. unstimulated, $^{\#}P$ < 0.05 vs. without inhibitor). (B) The phosphorylation pattern of phospho-Ser PKC substrates was evaluated by immunoblotting (n = 3).

Figure S2 (A and B) Direct effect of pharmacological drugs on angiogenesis. HMEC-1 were incubated with thrombin (0.05 U·mL^{-1})-stimulated platelets supernatants (Plt Sup) that were supplemented with increasing concentrations of COX-1 (aspirin, ASA), PKC (Gö6983), p38 (SB203580), ERK1/2 (U0126), Src kinases (PP1), or PI3K/Akt (Ly-294002) inhibitors after platelet stimulation. (C and D) Irreversible effect of aspirin. WPs were incubated with or without ASA (500 μM) for 30 min. Then, excess aspirin was removed (washed) or not (unwashed) and platelets were stimulated with thrombin (0.05 U·mL^{-1}) for 5 min. Subsequently, platelets supernatant collection, endothelial cell proliferation and tubule formation were determined (n = 3, $^{*}P$ < 0.05 vs. unstimulated, $^{\#}P$ < 0.05 vs. without inhibitor).

Figure S3 Effect of pharmacological inhibitors on endothelial viability. HMEC-1 were incubated with thrombin (0.05 U·mL^{-1})-stimulated platelets supernatants with or without the inhibitors of COX-1 (aspirin, ASA, 500 μM), PKC (Gö6983, 1 μM), p38 (SB203580, 25 μM), ERK1/2 (U0126, 10 μM), Src kinases (PP1, 5 μM), or PI3K/Akt (Ly-294002, 10 μM) pathways for 18 h. Then, cell viability was assessed by staining the cells with a mixture of acridine orange and ethidium bromide (100 < g·mL^{-1} each) to determine the percentage of cells that had undergone apoptosis (white bars) and necrosis (grey bars) by fluorescence microscopy. The addition of H2O2 (200 < M) were used as a positive control (n = 7, $^{\#}P$ < 0.05 vs. viable).

Table S1 Platelet-derived angiogenesis modulators measured by array. Results show mean of fluorescence intensity of each molecule (arbitrary units) (n = 2).