

ORIGINAL ARTICLE

Hyperthermia inhibits platelet hemostatic functions and selectively regulates the release of alpha-granule proteins

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Summary. *Background:* Hyperthermia is one of the main disturbances of homeostasis occurring during sepsis or hypermetabolic states such as cancer. Platelets are important mediators of the inflammation that accompanies these processes, but very little is known about the changes in platelet function that occur at different temperatures. *Objectives:* To explore the effect of higher temperatures on platelet physiology. *Methods:* Platelet responses including adhesion, spreading (fluorescence microscopy), $\alpha_{IIb}\beta_3$ activation (flow cytometry), aggregation (turbidimetry), ATP release (luminescence), thromboxane A_2 generation, alpha-granule protein secretion (ELISA) and protein phosphorylation from different signaling pathways (immunoblotting) were studied. *Results:* Preincubation of platelets at temperatures higher than 37 °C (38.5–42 °C) inhibited thrombin-induced hemostasis, including platelet adhesion, aggregation, ATP release and thromboxane A_2 generation. The expression of P-selectin and CD63, as well as vascular endothelial growth factor (VEGF) release, was completely inhibited by hyperthermia, whereas von Willebrand factor (VWF) and endostatin levels remained substantially increased at high temperatures. This suggested that release of proteins from platelet granules is modulated not only by classical platelet agonists but also by microenvironmental factors. The observed gradation of response involved not only antiangiogenesis regulators, but also other cargo proteins. Some signaling pathways were more stable than others. While ERK1/2 and AKT phosphorylation were resistant to changes in

temperature, Src, Syk, p38 phosphorylation and IkappaB degradation were decreased in a temperature-dependent fashion. *Conclusions:* Higher temperatures, such as those observed with fever or tissue invasion, inhibit the hemostatic functions of platelets and selectively regulate the release of alpha-granule proteins.

Keywords: alpha-granule secretion, hyperthermia, inflammation, platelet aggregation, platelets, temperature.

Introduction

The primary function of platelets is to initiate and maintain hemostasis. However, their function is not limited to thrombosis, and extends into physiological processes such as tissue repair, wound remodeling and antimicrobial host defense, or pathologic processes such as atherosclerosis, chronic ischemia or cancer [1–4]. In all these processes, platelets act as sentinels capable of responding instantly to chemical changes in their environment. The effect of platelets is protective and their absence can further exacerbate the injury to tissues [5]. A great variety of molecules are released from platelet granules upon activation, and these include prothrombotic substances, growth factors, chemokines and proteases. Many of the substances contained in platelets have physiologically opposing functions. It is not surprising that platelets secrete these proteins selectively, and only in response to tissue signals.

The role of platelets in inflammation is strongly influenced by reciprocal interaction between the cellular and humoral components of the microenvironment. As activated leukocytes and endothelial cells express adhesion molecules on their luminal surfaces, and as more inflammatory mediators (cytokines, chemokines, nitric oxide and reactive oxygen species) are secreted into the microenvironment, the sum of the various stimuli leads to either a positive platelet response (an enhancement of inflammation) or a negative one (an inhibition of inflammation) [6,7]. Although the typical attributes of the inflammatory microenvironment include acidosis, hypoxia and hyperthermia, there is little information as to the effect of these environmental stimuli on platelet function. It is generally

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accepted that hypothermia is associated with an increased risk of bleeding and may contribute to the morbidity and mortality in trauma and during complicated surgical procedures [8,9]. Nevertheless, the exact manner in which hypothermia affects platelet function remains controversial because low temperatures have been shown to both inhibit [8,9] or stimulate [10,11] thrombosis. In terms of platelet transfusion, lowering storage temperature has several benefits, among which is a bacteriostatic effect. However, platelet refrigeration remains impossible, because platelets desialylated due to chilling or sepsis are cleared in the liver by macrophages [12,13]. New evidence shows that galactosylation restores the circulation of short-term chilled murine platelets, but not of long-term refrigerated platelets [12].

Regarding hyperthermia, there is less reliable information about the impact of higher temperatures on the function of platelets. The available studies have focused on optimization of storage conditions for transfusional platelet products and on refining aggregation techniques, rather than on the biological consequences of this temperature sensitivity. The generally accepted conclusion from these studies is that storage of platelets at temperatures $< 37^{\circ}\text{C}$ induces platelet activation whereas temperatures $> 37^{\circ}\text{C}$ exert the opposite effect [14,15]. Yet, the *in vivo* responses of platelets to changes in temperature may be of far reaching consequence. A recent study focusing on the thermo-mechanical behavior of thoracic aortas in New Zealand White rabbits with different degrees of atherosclerosis, demonstrated that increases of corporal temperature, both locally or systemically, play an important role in increasing the risk of acute coronary syndromes [16]. The thermographic assessment of an atherosclerotic plaque (a surrogate indicator of the plaque metabolic state) has become an accepted predictive marker of vulnerable plaque at risk of rupture [17].

Similarly, heat stroke, a life-threatening state characterized by the rise of core body temperature above 40°C , is associated with a systemic inflammatory response leading to multiorgan dysfunction including abnormal coagulation [18]. Epidemiologic studies indicate that from 1979 to 1997, more than 7000 deaths in the United States were attributable to excessive heat, especially among the vulnerable population of the very young and elderly [19]. In Saudi Arabia, the incidence of heat stroke increases 10 times during the summer season and the mortality rate is estimated at 50% [18]. While the hemorrhagic manifestations and microvessel thrombosis are widespread and common in patients with severe heat stroke [20], the pathogenesis of this phenomenon is not fully understood.

Local changes in temperature are also typical during acute inflammatory processes of hypermetabolic states such as tumor growth. To improve the understanding of the local and systemic effects of increased temperature on platelet physiology, we have exposed human platelets to varying temperatures. We found that exposure of platelets to heat stress resulted in an impairment of platelet spreading, activation of $\alpha_{\text{IIb}}\beta_3$, aggregation, ATP release and thromboxane A_2 (TXA_2) formation. In addition, contrary to the present dogma that platelets

'degranulate' and release their contents *en masse*, the content of platelet alpha-granules was altered very selectively, and was dependent on inhibition of tyrosine kinases, p38 and nuclear factor-kappa B (NF-kappaB) signaling pathways.

Materials and methods

Cell preparation

Blood samples were obtained in plastic tubes containing sodium citrate (3.8%) from healthy donors who had not taken non-steroidal anti-inflammatory drugs for at least 10 days prior to sampling. The study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and received the approval of the institutional ethics committee. A written consent was obtained from all subjects. PRP was obtained by centrifugation at $180 \times g$ for 10 min. Washed platelet suspensions (WPs), prepared as previously described [21], were resuspended in Tyrode's buffer at a concentration of 4×10^8 per mL, unless otherwise stated. CaCl_2 (1 mM) was added before platelet stimulation.

Flow cytometry

To evaluate P-selectin and CD63 expression, platelets preincubated at different temperatures were stimulated, fixed and stained with a FITC-conjugated CD62P (anti-P-selectin antibody), anti-CD63 antibody or matched IgG isotype as a negative control (BD Biosciences, San Jose, CA, USA). Flow cytometry analysis was performed on a FACSCalibur flow cytometer using FCS EXPRESS V3 software, De Novo Software, Los Angeles, CA, USA. A similar technique was employed to evaluate CD61 expression (BD Biosciences) and $\alpha_{\text{IIb}}\beta_3$ integrin activation by using FITC-conjugated PAC1 (BD Biosciences) or Alexa488-conjugated fibrinogen (Invitrogen, Eugene, OR, USA). Results are expressed as percentage of positive cells.

Platelet adhesion and spreading on fibrinogen

WPs (5×10^7 per mL) were plated on fibrinogen ($100 \mu\text{g mL}^{-1}$)-coated slides (Sigma, St. Louis, MO, USA) for 20 min. Adhered platelets were fixed, permeabilized, stained with TRITC-Phalloidin, mounted, and visualized using fluorescent microscopy [22].

Platelet aggregation and ATP release

Aggregation and ATP release were measured simultaneously in a Lumi-aggregometer (Chrono-Log, Havertown, PA, USA) as previously described [22]. Aggregation was induced by thrombin, arachidonic acid (AA), ADP (Sigma) or collagen (Nycomed Pharma, Unterschleibheim, Germany). ATP levels were calculated at the end of the assay by adding a known amount of ATP (Sigma). Results are expressed as the percentage of maximal light transmission.

Measurement of thromboxane B_2 (TXB₂) release

WPs were incubated with ADP for 5 min in an aggregometer chamber stirring at 1000 rpm. The addition of ice-cold PBS containing EDTA (2 mM) and aspirin (500 μ M) was used to stop the reaction. The samples were centrifuged and TXB₂ was measured in the supernatants using an ELISA kit from Cayman Chemical (Ann Arbor, MI, USA).

Measurement of von Willebrand factor (VWF), vascular endothelial growth factor (VEGF), and endostatin levels

WPs were stimulated with thrombin for 5, 15 or 30 min. The reaction was stopped by the addition of PBS containing prostacyclin (75 nM) (Cayman). Samples were centrifuged twice at $1100 \times g$ for 5 min and then at $9300 \times g$ for 5 min and supernatants stored at -80°C until assayed for VWF (Dako, Glostrup, Denmark), VEGF and endostatin (RayBio-tech Inc, Norcross, GA, USA) levels by ELISA.

Immunoblotting

WPs were lysed ($1\text{--}2 \times 10^9$ per mL) 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 blocking, the membranes were incubated overnight at 4°C with primary antibodies (pSrc-Tyr416, pSyk-Tyr525/526 and pp38-Thr180/Tyr182 from Cell Signaling, Danvers, MA, USA, pERK E-4 and pAkt1/2/3-Ser473 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-quantitated 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.

Statistical analysis

Results were expressed as means $\pm$ SEM and analyzed by one-way analysis of variance followed by the Newman-Keuls multiple comparison test to determine significant differences between groups. *P*-values lower than 0.05 were considered statistically significant.

Results

Inside-out platelet signaling is decreased at high temperature

In order to improve the understanding of the effect of hyperthermia on platelet physiology, several responses resulting from platelet activation were evaluated at different temperatures ranging from the physiological 37 to 42°C . The interaction of a soluble platelet agonist with its receptor leads to

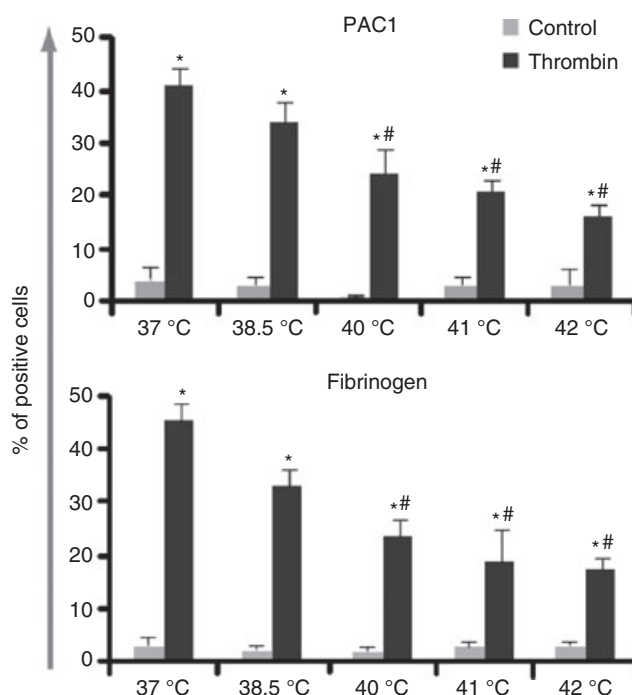


Fig. 1. Heat exposure negatively regulated thrombin-induced $\alpha_{IIb}\beta_3$ activation. WPs were incubated at 37, 38.5, 40, 41 or 42°C for 10 min and then stimulated with thrombin (0.05 U mL^{-1}) for 5 min. Analysis of PAC-1 and fibrinogen binding was performed by flow cytometry ($n = 6$). **P* < 0.05 vs. unstimulated control; #*P* < 0.05 vs. thrombin at 37°C .

a cascade of intracellular events, broadly classified as inside-out signaling. The subsequent induction of conformational changes of the $\alpha_{IIb}\beta_3$ integrin, leads to exposure of neoepitopes and fibrinogen binding [23]. We found that thrombin-induced $\alpha_{IIb}\beta_3$ activation, as reflected by lower PAC1 and fibrinogen binding, is down-regulated in a temperature-dependent fashion (Fig. 1). To rule out that the decreased fibrinogen binding was due to a temperature-dependent alteration of either the fibrinogen molecule or the fluorophore, Alexa 488-conjugated fibrinogen alone was preincubated at 37, 40 or 42°C for 10 min, before adding it to the platelets stimulated with thrombin at room temperature. The percentage of fibrinogen binding obtained after staining with the prewarmed fibrinogen molecule was similar to the values obtained with a non-prewarmed one (Fig. S1A). The same control experiment was performed with the FITC-conjugated PAC1 antibody, and, again, neither the FITC fluorochrome nor the antibody was affected by the temperature increases (data not shown). In addition, no changes in fibrinogen binding were observed when platelets at RT were stimulated with prewarmed ($37\text{--}42^\circ\text{C}$) thrombin (data not shown), indicating that the decreased activation of $\alpha_{IIb}\beta_3$ integrin is due to a defect in platelet function rather than a reagent alteration/degradation.

Effect of temperature on platelet adhesion and spreading

The development of lamellipodia and filopodia was used as a surrogate for the interaction between glycoprotein $\alpha_{IIb}\beta_3$ and its

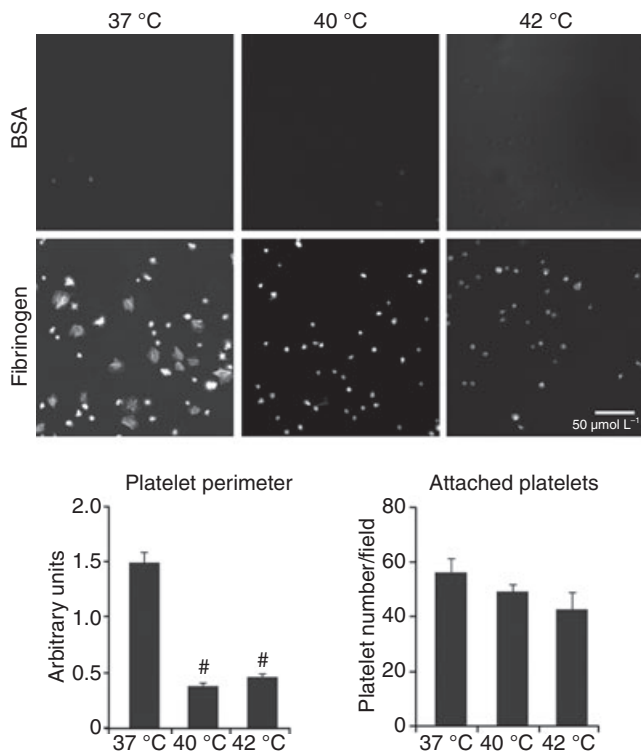


Fig. 2. High temperature inhibits platelet spreading. WPs were incubated at 37, 40 or 42 °C for 10 min and then plated on BSA- or fibrinogen-coated slides and stained with TRITC-Phalloidin. Platelet spreading was visualized under fluorescent microscopy (original magnification 600×). Platelet perimeter and number per field were measured using IMAGE-PRO software ($n = 4$). $P < 0.01$ vs. 37 °C.

binding partners. Platelet attachment and spreading is understood to be a direct consequence of the outside-in signaling triggered by $\alpha_{IIb}\beta_3$ interaction with its ligand. It represents one of the essential steps for the initiation of hemostasis and thrombosis [23]. Even though higher temperatures did not dramatically affect the adhesion of platelets to fibrinogen-coated surfaces, temperatures of 40 and 42 °C completely inhibited the platelet spreading and lamellipodia formation seen at the physiological temperature of 37 °C (Fig. 2).

Inhibition of platelet aggregation, ATP release and TXA₂ formation by high temperatures

Having shown that hyperthermia decreased fibrinogen and PAC1 binding, we went on to examine the effect of temperature on platelet aggregation. Figure 3 reveals a temperature and agonist sensitive pattern of platelet aggregation. At 40 °C, the low concentrations of arachidonic acid (AA) and ADP have close to a full effect on platelet aggregation (Fig. 3B,C), whereas thrombin, collagen and higher AA concentrations reach this level of inhibition only at a temperature of 42 °C (Fig. 3A,B). The degree of inhibition of platelet aggregation was in all cases associated with the ATP release. As is shown in Fig. 3(C), the marked reduction in platelet aggregation by ADP (2 μ M) was accompanied by a complete inhibition of

ATP release. In the same line, the moderate inhibition of the aggregation response induced by thrombin, collagen or AA (0.5 mM) at 40 °C was parallel to a moderate decrease in ATP release (data not shown).

Although the inhibitory effect of the 40 °C temperature on platelet aggregation could be overcome by higher concentrations of ADP (5 μ M), even in the absence of TXA₂ and ATP (data not shown), this gain achieved by a higher dose of ADP was lost at 42 °C (Fig. 3C). The more potent agonists of platelet aggregation, such as thrombin and high collagen concentrations, were less affected by temperature. Thus, there was a very graded reaction to temperature for each of the individual agonists, as well as a very typical and distinct pattern of response by the various tested agonists.

Differential regulation of platelet alpha-granule release by temperature

The activation of various signaling cascades during platelet activation ultimately leads to release of the contents of dense and alpha granules. The generally accepted paradigm that 'degranulation' of platelet alpha granules leads to secretion of most substances *en mass* is presently being revised to reflect a more sequential and selective release of granular content in tissues [24–26]. Platelet alpha granule release at the site of vascular injury is now thought to be regulated by many biological processes including inflammation [27]. Our experiments were in agreement with this new understanding. While a remarkable release of all of these proteins normally stored in platelet granules can be induced by thrombin at 37 °C, higher temperatures of 40 and 42 °C temper this dramatic release (Fig. 4). There was a significant variability in the grade of the response. For example, exposure of P-selectin on the platelet surface and the release of VEGF was dramatically inhibited at 40 °C and almost completely suppressed at 42 °C. In contrast, the inhibition of thrombin-induced VWF or endostatin release was less dramatic and more gradual (Fig. 4A). In fact, while P-selectin and VEGF levels triggered by thrombin at 42 °C are almost no different from those found in unstimulated platelets, VWF and endostatin levels are still significantly raised by thrombin.

Because during the later secretory stages platelets undergo contraction and increased granule release into the medium, we next examined the effect of temperature after 15 and 30 min thrombin activation. Among the different proteins evaluated under these conditions, only the release of VEGF was slightly increased after 30 min compared with 5 min stimulation. While the secretion of P-selectin and VEGF was markedly decreased with higher temperatures, the inhibition of VWF and endostatin was less dramatic (Fig. 4B). Because P-selectin is found not only in alpha but also in dense granules [28], we analyzed the expression of CD63, an ubiquitous protein found mainly in the membrane of lysosomal, dense granules but also in alpha granules to some extent [29]. We found that like P-selectin, hyperthermia negatively regulates CD63 expression (Fig. S1B).

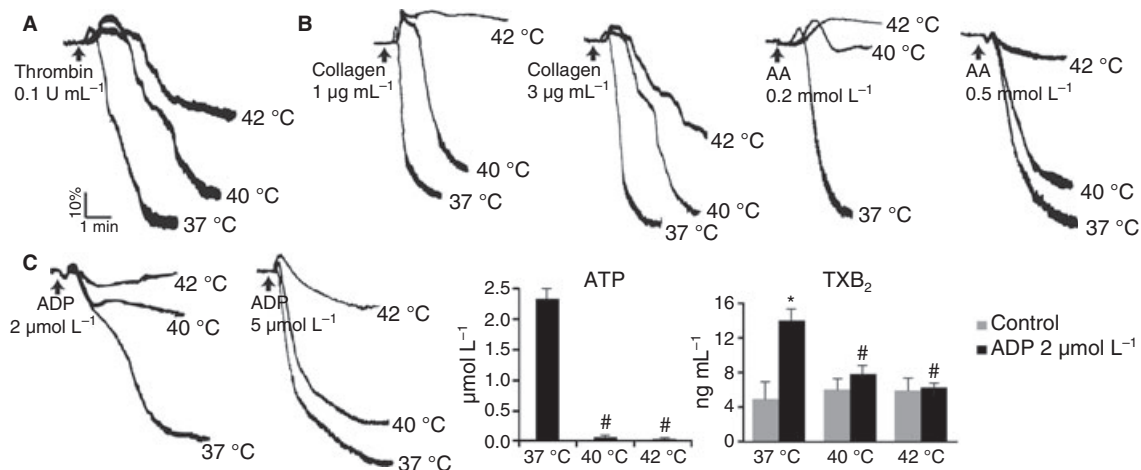


Fig. 3. Inhibition of platelet aggregation, ATP release and TXB₂ formation at high temperatures. (A) WPs or (B) PRP were incubated at 37, 40 or 42 °C for 10 min and then aggregation was induced by the indicated agonists. Graphics are representative of four independent experiments. (C) PRP was incubated at 37, 40 or 42 °C for 10 min and then stimulated with ADP. Aggregation and ATP release were determined simultaneously in a Lumi-aggregometer ($n = 4$). After 5 min stimulation, supernatants were collected to measure TXB₂ levels by ELISA. * $P < 0.05$ vs. unstimulated control. $P < 0.05$ vs. thrombin at 37 °C ($n = 3$).

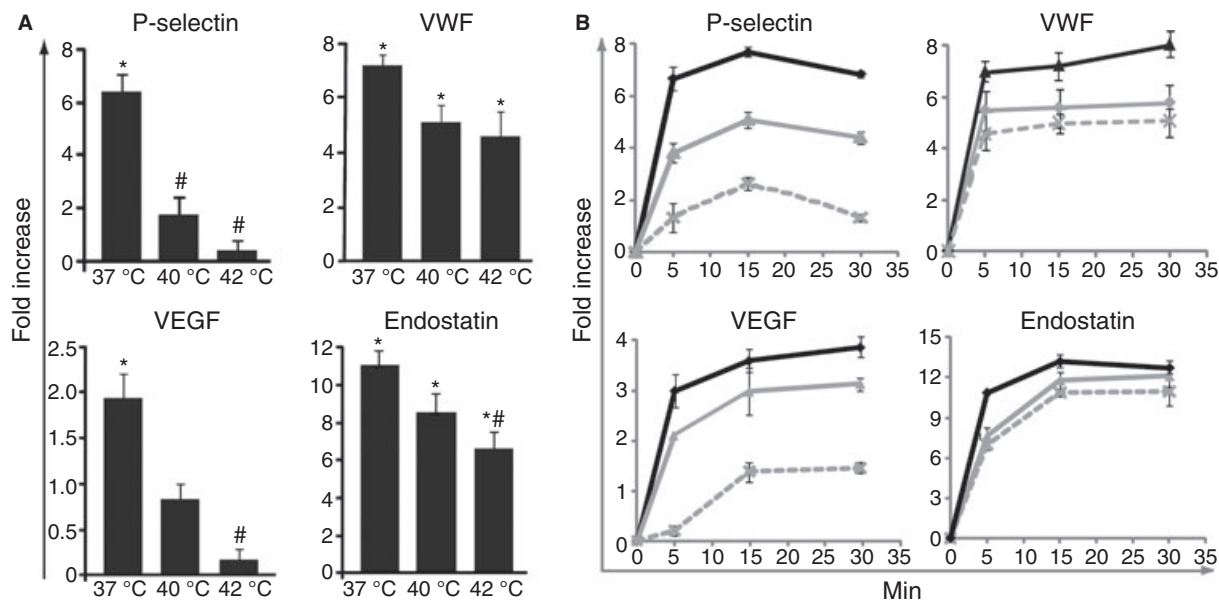


Fig. 4. Effect of hyperthermia on platelet alpha-granule secretion induced by thrombin. WPs were preincubated at 37 (dark line), 40 (grey line) or 42 °C (dashed line) for 10 min and then stimulated with thrombin (0.05 U mL⁻¹) for 5 min (A) or at the indicated time (B). P-selectin was detected by flow cytometry ($n = 10$). VWF, VEGF and endostatin levels in the supernatants were quantified by ELISA ($n = 5$). As the basal levels of each protein release in unstimulated controls were not affected by the temperature, results are expressed as fold increase vs. unstimulated controls at the same temperature. * $P < 0.05$ vs. unstimulated control; $P < 0.05$ vs. thrombin at 37 °C.

Effect of temperature on platelet signaling

To explore the molecular changes that accompany the temperature-induced changes in platelet function, we measured the degree of phosphorylation of proteins participating in the activation of different signaling pathways. We found that thrombin caused a robust phosphorylation of all kinases tested (ERK1/2, p38, AKT, Syk and Src) in a concentration-dependent manner. While the degree of thrombin-induced

phosphorylation was not modified by temperature in the case of pERK1/2 and pAKT, p38, Syk and Src phosphorylation levels were significantly reduced at 40 °C and completely abolished at 42 °C (Fig. 5A,B). It has been shown that heat shock proteins are present in platelets and several members of this family mediate platelet function such as adhesion, aggregation and granule release [30]. Considering that phosphorylation of HSP27 is downstream to and catalyzed by p38 MAPK [31], we examined the effect of temperature on

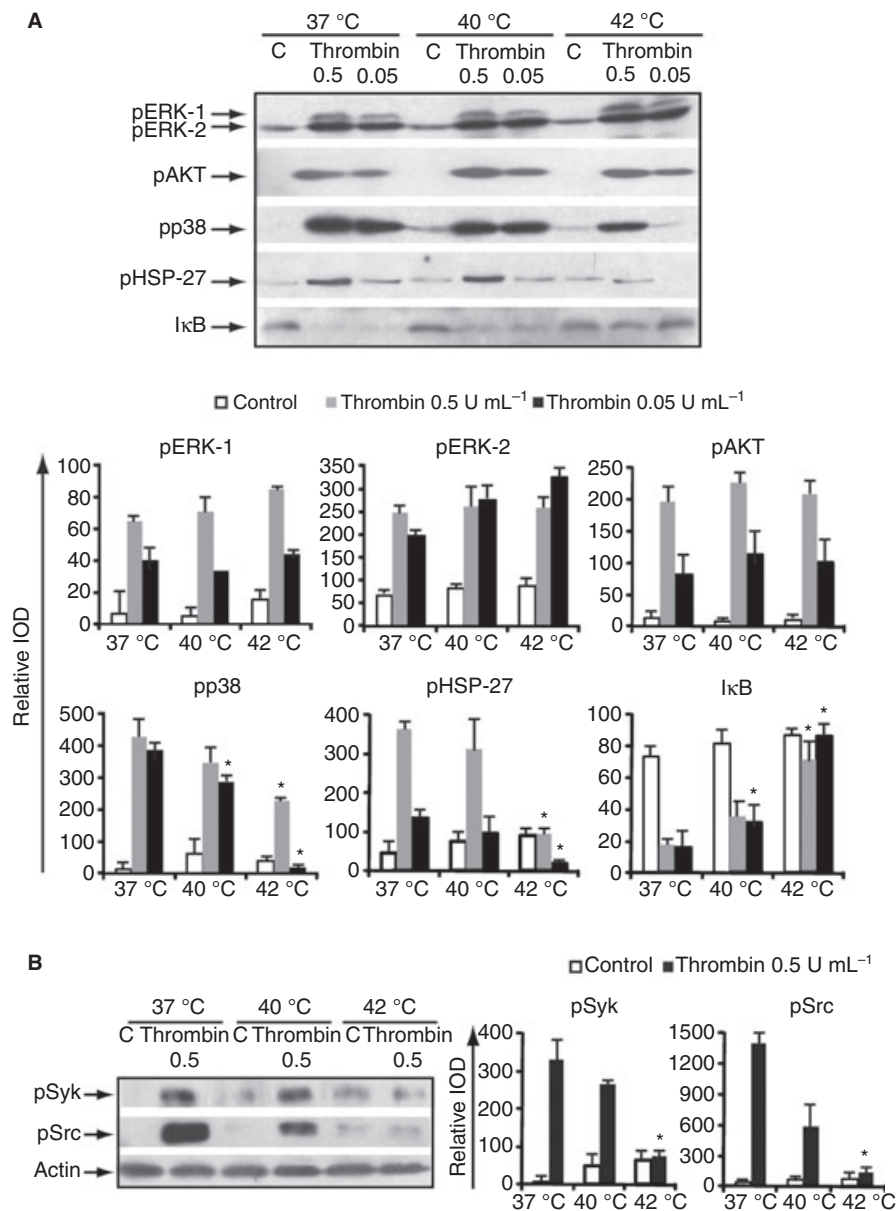


Fig. 5. Effect of temperature on platelet signaling. WPs were preincubated at 37, 40 or 42 °C for 10 min. (A) WPs were stimulated with the indicated concentration of thrombin for 5 min and lysates were immunoblotted with anti-pp38, pERK1/2, pHSP27 or pAKT antibody. (B) WPs were stimulated with thrombin for 2 min and lysates were immunoblotted with anti-pSyk and pSrc antibody. Each membrane was reprobed with anti-actin antibody to calculate the relative IOD using GEL-PRO software ($n = 3-5$). * $P < 0.05$ vs. thrombin at 37 °C.

pHSP27. As expected, thrombin-induced phosphorylation of HSP27 was decreased in platelets exposed to high temperature (Fig. 5A).

We have recently shown that platelets express the NF-kappaB machinery and that the activation of this transcription factor is involved in platelet activation [21]. We now observed that similarly to p38, I-kappaB degradation, a measure of NF-kappaB activation, was dampened as temperature increased (Fig. 5A).

In view of these findings, we chose to analyze the role of p38 and the recently described NF-kappaB pathway in the temperature-mediated differential release of alpha-granule

contents by using specific inhibitors of p38 (SB203580) and NF-kappaB (RO1069920) [21] at concentrations that have been confirmed to completely inhibit their phosphorylation (Fig. S1C). We found that the individual inhibitors failed to produce a statistically significant inhibition of the thrombin-induced release of alpha-granule proteins (Fig. 6), but the combination of p38 and NF-kappaB inhibition led to a statistically significant inhibition of P-selectin expression, VEGF release, VWF and endostatin (Fig. 6). Conversely, inhibition of pERK1/2 or PI3K/pAKT (by U0126 or LY294002 and Wortmannin, respectively) failed to regulate the release/expression of alpha-granule proteins (data not

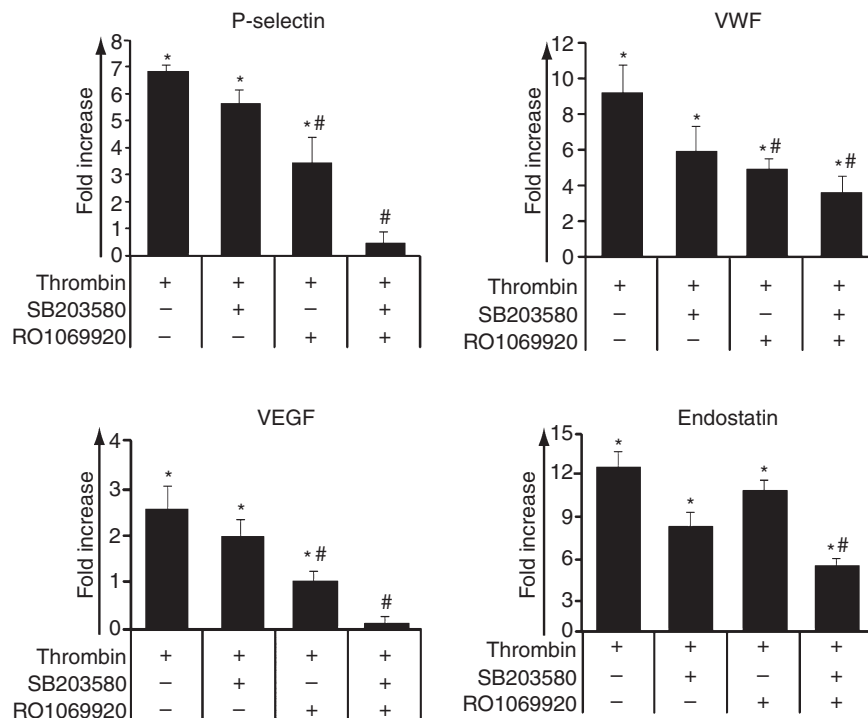


Fig. 6. Inhibition of p38 and NF-kappaB pathways mimics the effect of heat exposure on platelet alpha-granule secretion. WPs were preincubated with SB203580 (25 μ M), RO1069920 (6 μ M) or the combination of both for 10 min and then stimulated with thrombin (0.05 U mL⁻¹) for 5 min. P-selectin was detected by flow cytometry ($n = 5$). VWF, VEGF and endostatin levels in the supernatants were quantified by ELISA ($n = 4$). Results are expressed as fold increase vs. unstimulated controls. * $P < 0.05$ vs. unstimulated control; # $P < 0.05$ vs. thrombin alone.

shown) although they efficiently suppressed phosphorylation of ERK1/2 or AKT (Fig. S1C).

Discussion

Platelet homeostasis and function is maintained within a very narrow physiological range of temperature. The understanding, however, suggests that this 'sensitivity' of platelets to temperature has more to do with platelet fragility than with a regulation of their function. We would like to propose that the sensitivity of platelets to external stimuli such as changes in temperature may be an evolutionally beneficial mechanism of facilitating a selective and sequential release of factors needed for inflammatory and angiogenic processes during development, tissue regeneration, wound healing or tumor growth. Our studies provide early evidence that platelet exposure to temperatures higher than the physiological temperature of 37 °C but within the pathological range of *in vivo* events, inhibit aggregation in a dose-dependent fashion. The pattern of platelet aggregation response to temperature is specific to individual agonists (Fig. 3B). Smaller rises in temperature (at 40 °C) and low doses of arachidonic acid and ADP result in a close to maximal inhibitory effect on platelet aggregation, suggesting that these acute inflammatory responders are responsible for early platelet response. In contrast, thrombin and collagen reach this higher level of aggregation inhibition only at tempera-

tures of 42 °C. Of note is that thrombin and collagen are deposited in tissues in late stages of tissue organization and in chronic wounds and this differential response to different agonists at different temperatures provides a means to fine-tune a tissue response. The graded response to hyperthermia may provide clues as to the meticulous orchestration of early and late stages of wound healing.

Several other platelet responses, including spreading, PAC1 and fibrinogen binding, and ATP release were similarly regulated in resting and thrombin-activated platelets exposed to varying physiologically relevant temperatures. Interestingly, while preincubation of thrombin or fibrinogen at 40 or 42 °C failed to modify the platelet ability to bind fibrinogen, other thrombin-induced parameters such as phosphorylation of ERKs and AKT were not modified by temperature variations (see Fig. 5), which indicated that hyperthermia was affecting platelet function rather than agonists or reagent quality/integrity.

The fact that hyperthermia dampened the hemostatic function of platelets would suggest that high fever should lead to hemorrhage. Although the mechanisms of bleeding have been extensively explored in the hemorrhagic fever-related diseases, no deep analysis regarding the linkage between platelet function and body temperature has been performed in those patients. However, there is evidence to suggest that an abnormal platelet function is found on patients with fever because it has been reported that while the threshold for

prophylactic transfusions in cancer patients can safely be set at 5×10^9 per L in the absence of fever, the threshold should be at least 20×10^9 per L in those patients with fever $> 38^\circ\text{C}$ or under hyperthermia therapy to avoid bleeding [32].

The newly revised paradigms of platelet selective 'alpha granule degranulation' [25,33], and the concept that a sequential platelet granule release at the site of vascular injury [26,27] contributes to the up- or down-regulation of processes such as inflammation and angiogenesis [34], were supported by our findings. We showed that exposure of P-selectin on the platelet surface and the release of VEGF were dramatically inhibited at 40°C and almost completely suppressed at 42°C (Fig. 4), suggesting that these acute phase reactants are early and transient responders. In contrast, the inhibition of thrombin-induced VWF or endostatin release at 40 and 42°C was much less dramatic, suggesting a lingering pattern of their release. A possible explanation for this differential response is that higher temperatures such as those occurring in high metabolic states (burns, wounds or tumors), may require ongoing activity of stromal (VWF) and inhibitory proteins (endostatin), whereas chemoattractants (P-selectin) and inducers of angiogenesis (VEGF) are required for the initial induction of inflammatory chemotaxis and angiogenesis. In addition, it might be possible that released granule components have (transient) interaction with binding partners on the surface of the open canalicular system.

Interestingly, it has been shown previously that VWF colocalizes with endostatin but not with VEGF, and that a distinct and separate organization exists for the different proteins within the alpha granules [25]. Our findings indicate that the differential granule release is regulated not only by tissue platelet agonists but also by changes in microenvironmental factors such as temperature. The different regulatory effect of temperature on P-selectin expression and VWF release is intriguing because both proteins appeared to be stored in similar alpha granules. Using 3D analysis and combined immune-electronmicroscopy characterization, van Nispen tot Pannerden *et al.* [29] suggested that besides the existence of morphologically different alpha-granule subtypes (tubular and spherical), a complex compartmentalization and a high spatial segregation of cargo exist within individual α -granules. This leads us to speculate that soluble and membrane-bound proteins appear highly compartmentalized within individual alpha granules and their release may be governed by different mechanisms. In fact, we found that CD63, another granule membrane-bound protein followed a temperature-inhibition pattern similar to P-selectin (Fig. S1C). To what extent differences in granule morphology and spatial protein segregation within individual alpha granules contribute to the observed temperature-dependent differential secretory activity of platelet alpha granules contents remains to be determined.

Local hyperthermia is used to enhance the effectiveness of cancer treatments such as ionizing radiation [35]. Platelets are crucial regulators of tumor vascular homeostasis because they deliver angiogenesis-modulating factors to the tumor site [36]. Our results showed that high temperatures tempered the release

of VEGF while sparing the secretion of the anti-angiogenic endostatin. The control of platelet activation might therefore be one of the physiological mechanisms involved in the local hyperthermia effect during radiation therapy.

A deeper analysis of the mechanisms involved in the hyperthermia-mediated inhibition of platelet aggregation revealed that lack of aggregation after ADP stimulation was associated with a robust decrease in TXA_2 generation and in p38 phosphorylation. It is well known that pp38 activates cyclooxygenase-1 and -2 and thromboxane synthase [37], suggesting that the cyclo-oxygenase-1 and/or thromboxane synthase may be the main targets of the hyperthermia-mediated effects on AA metabolism.

In addition to the extensively studied PI3K/AKT pathway in platelets [38,39], the multiple MAPK and Src family kinases (SFKs) involving pathways have now become the focus of growing research. Evidence now exists that ERK1/2, p38 and JNK1 are present in platelets and become phosphorylated in response to platelet stimulation with thrombin, ADP and collagen among others [40]. Platelets express at least six SFKs, among which Fyn and Lyn are involved in the activation of platelets following binding of collagen or laminin to the platelet-specific GPVI/Fc receptor (FcR) c-chain complex. Another important platelet activation pathway that involves SFKs, specifically Src itself and Fyn, is that of outside-in signaling by the platelet-specific integrin, $\alpha_{\text{IIb}}\beta_3$ [41]. To study the molecular changes associated with the physiological hyperthermia effect on platelet biology, we analyzed the aforementioned pathways in platelets incubated at 37 , 40 and 42°C . We have found that even though thrombin-induced phosphorylation of ERK1/2 and AKT was not modified by higher temperatures, pSrc, pSyk and p38 activation was completely abolished. Similar results were reported in synovocyte, where short-term hyperthermia prevented IL-1 β -induced p38 phosphorylation but not pERK1/2 or pAKT [42]. Moreover, heat-mediated inhibition of the small HSP27 phosphorylation, one of the main targets of active p38 in human platelets, further confirmed the negative regulation of high temperature on the p38 signaling pathway [30].

Although platelets are anucleate cellular structures, they express several transcription factors that exert non-genomic functions, including the positive and negative regulation of platelet activation. We [21] have along with others [43,44] previously demonstrated that NF-kappaB was expressed in platelets and its pharmacological inhibition by RO1069920 suppressed different platelet activation responses including P-selectin expression and VWF release. A combined pharmacological blockade of pp38 and NF-kappaB by SB203580 and RO1069920, respectively, mimicked the effect of temperature on alpha-granule release. The thrombin-induced P-selectin expression and VEGF release were completely suppressed by the synergistic action of the dual pathway inhibition, whereas VWF and endostatin levels remained elevated, suggesting that the differential effect of temperature on alpha-granule release may be linked to a selective inhibition of p38 and NF-kappaB signaling pathways.

In conclusion, the sum of effects of physiologically relevant hyperthermia on platelets presented above suggests that the sensitivity of platelets to temperature may be of pathophysiological relevance. We demonstrated that exposure to higher temperatures had a negative effect on platelet hemostatic function, and that the release of alpha-granule contents varied with increasing temperatures. This graded response may be reflective of a carefully orchestrated tissue-controlled response by platelets rather than platelet fragility. The recognition of this phenomenon may aid future development of therapeutic agents, which are presently tested at ambient temperatures.

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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:

Figure S1.

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