Sustained hypothermia accelerates microvascular thrombus formation in mice

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Lindenblatt, Nicole, Michael D. Menger, Ernst Klar, and Brigitte Vollmar. Sustained hypothermia accelerates microvascular thrombus formation in mice. Am J Physiol Heart Circ Physiol 289: H2680–H2687, 2005. First published August 12, 2005; doi:10.1152/ajpheart.00425.2005.—Cold is supposed to be associated with alterations in blood coagulation and a pronounced risk for thrombosis. We studied the effect of clinically encountered systemic hypothermia on microvascular thrombosis in vivo and in vitro. Ferric chloride-induced microvascular thrombus formation was analyzed in cremaster muscle preparations from hypothermic mice. Additionally, flow cytometry and Western blot analysis was used to evaluate the effect of hypothermia on platelet activation. To test whether preceding hypothermia predisposes for enhanced thrombosis, experiments were repeated after hypothermia and re-warming to 37°C. Control animals revealed complete occlusion of arterioles and venules after 742 ± 150 and 824 ± 172 s, respectively. Systemic hypothermia of 34°C accelerated thrombus formation in arterioles and venules (279 ± 120 and 376 ± 121 s; P < 0.05 vs. 37°C). This was further pronounced after cooling to 31°C (163 ± 57 and 281 ± 71 s; P < 0.05 vs. 37°C). Magnitude of thrombin receptor activating peptide (TRAP)-induced platelet activation increased with decreasing temperatures, as shown by 1.8- and 3.0-fold increases in mean fluorescence after PAC-1 binding to glycoprotein (GP)IIb-IIIa and 1.6- and 2.9-fold increases of fibrinogen binding on incubation at 34°C and 31°C. Additionally, tyrosine-specific protein phosphorylation in platelets was increased at hypothermic temperatures. In re-warmed animals, kinetics of thrombus formation were comparable to those in normothermic controls. Concomitantly, spontaneous and TRAP-enhanced GP IIb-IIIa activation did not differ between re-warmed platelets and those maintained continuously at 37°C. Moderate systemic hypothermia accelerates microvascular thrombosis, which might be mediated by increased GP IIb-IIIa activation on platelets but does not cause predisposition with increased risk for microvascular thrombus formation after re-warming.

Bleeding diathesis with prolongation of bleeding times has been reported in a variety of clinical settings associated with systemic hypothermia (19, 28, 36). In trauma patients hypothermia has been presumed to adversely affect blood coagulation (7, 31). In line with this, in vitro studies suggested that perioperative hypothermia may aggravate surgical bleeding by impairing platelet P-selectin expression and thromboxane release as well as by reducing activity of coagulation factors (24, 32).

In clear contrast to these reports on hypothermia-related bleeding diathesis, cold-related pathology, such as the seasonal increase in thromboembolic disease in winter, is well recognized (8, 9, 30). Cold is associated with peripheral vasoconstriction and increased cardiac output, blood pressure, and circulating norepinephrine, all of which enhance the risk for cardiac events and coronary heart disease mortality. The fact that in regions without temperature extremes a seasonal variation in myocardial infarction is absent (20) emphasizes the ambient air temperature as an environmental risk factor. Seasonal changes in temperature further influence blood rheology with increase of viscosity of blood and resistance of red blood cells to deform as temperature is lowered, contributing in a major way to impaired circulation in the cold (21, 26). The Caerphilly prospective heart disease study, comprising data on the association of air temperature and risk factors for ischemic heart disease from up to 2,036 men, indicates that the most important effect of a fall in temperature seems to be on the hemostatic system, with an increase of fibrinogen and a decrease in the fibrinolysis-inhibiting α2-macroglobulin (8). Moreover, platelet count and platelet aggregation have been shown in vitro to increase at hypothermic temperatures (8, 10, 17). Unintentional perioperative hypothermia is associated with postoperative myocardial ischemia, additionally underlining a potential prothrombogenic effect of low body temperature (12).

To further address this issue, we evaluated hypothermia-induced platelet response in vitro using temperatures of 34°C and 31°C, which are likely to be encountered during major surgery, multiple trauma, cold exposure, and the neonatal period. We additionally assessed kinetics of microvascular thrombus formation in an in vivo model of hypothermic mice. To test whether preceding hypothermia predisposes to thrombus formation, in vitro and in vivo experiments were repeated on hypothermia and subsequent re-warming to 37°C.

MATERIALS AND METHODS

Mouse cremaster muscle preparation. On approval by the local government, all experiments were carried out in accordance with German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). Male C57BL/6J mice with a body weight of 20–25 g were anesthetized by an intraperitoneal injection of ketamine (90 mg/kg body wt) and xylazine (25 mg/kg body wt), and a polyethylene catheter was placed into the right jugular vein for application of fluorescent dyes.

For the study of vascular thrombus formation, we used the opened cremaster muscle preparation, as originally described by Baez (1) in rats and transferred by our group (22, 42) to mice. Before preparation of the cremaster muscle, animals were placed on a heating pad coupled to a rectal probe. A midline incision of the skin and fascia was made over the ventral aspect of the scrotum and extended up to the inguinal fold and to the distal end of the scrotum. The incised tissues

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were retracted to expose the cremaster muscle sack, which was maintained under gentle traction to carefully separate the remaining connective tissue by blunt dissection from around the cremaster sack. The cremaster muscle was then incised, avoiding cutting of the larger anastomosing vessels. Hemostasis was achieved with 5-0 threads, also serving to spread the tissue. After dissection of the vessel connecting the cremaster and the testis, the epididymis and testis were put to the side of the preparation. The preparation was performed on a transparent pedestal to allow microscopic observation of the cremaster muscle microcirculation by both transillumination and epi-illumination techniques.

After the preparation of the cremaster muscle, the animals were allowed to recover from surgical preparation for 15 min. Thrombus formation was then induced in randomly chosen venules (n = 1 or 2 per preparation) and arterioles (n = 1 or 2 per preparation).

**In vivo thrombosis model.** After intravenous injection of 0.1 ml of 5% FITC-labeled dextran (mol wt 150,000; Sigma-Aldrich, Munich, Germany) and subsequent circulation for 30 s, the cremaster muscle microcirculation was visualized by intravital fluorescence microscopy with a Zeiss microscope (Axiovert 25; Zeiss Jena, Germany). The microscopic procedure was performed at a constant room temperature of 21–23°C. The epi-illumination setup included a 100-W HBO mercury lamp and an illuminator equipped with a blue filter (450- to 490-nm excitation and >520-nm emission wavelengths). Microscopic images were recorded by a charge-coupled device video camera (FK 6990A-IQ; Pieper, Berlin, Germany) and stored on videotapes for offline evaluation (S-VHS Panasonic AG 7350-E; Matsushita, Tokyo, Japan). With a ×20 water immersion objective (Achromplan ×20/0.50 W; Zeiss) blood flow was monitored in individual arterioles (diameter range 30–50 μm) and venules (diameter range 60–80 μm), followed by superfusion with 25 μl of ferric chloride (12.5 mM; Sigma) for induction of microvascular thrombosis (6, 22). Complete vessel occlusion was assumed when blood flow ceased for >60 s because of thrombotic occlusion. Because rapid spreading of ferric chloride solution allowed us to study only 1 or 2 arterioles and venules within each preparation, both left and right cremaster muscles were prepared for analysis of thrombotic vessel occlusion within each animal.

Analysis included the time periods until first standstill of perfusion and sustained cessation of blood flow due to complete vessel occlusion. Additionally, a red blood cell velocity profile was determined to characterize the kinetics of microvascular thrombus formation. Microcirculatory analysis further included the determination of vessel diameter and blood cell velocity before thrombus induction with a calculation of vascular wall shear rates based on the Newtonian definition γ = 8 × V/D, with V representing the red blood cell center line velocity divided by 1.6 according to the Baker-Wayland factor (2) and D representing the individual inner vessel diameter.

**Experimental design.** Immediately after induction of anesthesia, animals were placed on a customized platform comprising a heating pad to facilitate microscopy of the cremaster muscle. Temperature was controlled by a rectal probe and maintained at 37°C (n = 6), 34°C (n = 5), and 31°C (n = 5). Because accurate maintenance of the animals’ core body temperature was a prerequisite for this study, the examiner was not blinded to animal temperature.

The assumption that the rectal temperature equaled the core body temperature was confirmed as described above. In an additional set of experiments, mice were kept hypothermic at either 34°C (n = 4) or 31°C (n = 4) for at least 30 min, followed by rewarming to 37°C and subsequent thrombus induction.

**Human blood collection and platelet-rich plasma preparation.** Written informed consent of all volunteers was obtained for blood drawing. For in vitro tests of platelet function, blood from a total of seven healthy volunteers was drawn from the left cubital vein with a 21-gauge needle into 5-ml S-Monovettes 9NC (Sarstedt, Nümbrecht, Germany) (1:10 citrate vol/vol). Despite differences in size, number, and ultrastructural morphology, human and murine platelets have been shown to exert similar organelle and glycoprotein (GP) subcellular distribution (37). The GPIIb-IIIa receptor in particular exerts comparable functions during platelet activation and aggregation in humans and mice (40). Although species differences cannot be completely excluded, the use of human platelets is justifiable and obvious because of their simple accessibility and ease of handling for flow cytometric studies.

After centrifugation for 15 min at 110 g and room temperature (GS-6R Centrifuge; Beckman Coulter, Fullerton, CA), platelet-rich plasma (PRP) was transferred in a separate tube. Platelet count was assessed with a cell counter (Sysmex KX-21; Sysmex, Norderstedt, Germany) and adjusted to 2 × 10^9/ml by dilution with PBS. In parallel, aliquots of whole blood were processed for the flow cytometric study of thrombin receptor activating peptide (TRAP)/N-formylmethionyl-leucyl-phenylalanine (FMLP)-induced platelet-leukocyte aggregates (see below).

**Platelet exposure to TRAP and flow cytometric analysis of P-selectin expression.** GPIIb-IIIa activation, and fibrinogen binding. After 30 min of resting in a 37°C water bath to eliminate isolation-induced platelet activation, 50 μl of platelet suspensions was incubated for 30 min in water baths at the maintained temperature of 37°C, 34°C, or 31°C, followed by exposure to TRAP (2.5 mM) and incubation with saturating amounts of the appropriate antibody or fluorescence-labeled human fibrinogen. Platelet suspensions were kept for an additional 30 min in the respective water baths in the dark. Platelets were then rapidly cooled on ice and diluted with 1 ml of 4°C 1% paraformaldehyde in PBS (Cell Fix; Becton Dickinson, Heidelberg, Germany). After fixation was completed, platelets were centrifuged at 300 g for 4 min at 4°C and washed twice with PBS. The supernatant fraction was decanted, and the pellet was resuspended in PBS for flow cytometry. Expression of P-selectin on platelets was investigated by direct immunofluorescence using a monoclonal anti-human FITC-coupled P-selectin antibody (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:50 (vol/vol) with staining medium (0.1% sodium azide and 2% fetal calf serum in PBS). In an additional set of experiments a FITC-coupled PAC-1 antibody (Becton Dickinson Biosciences) directed against the activated conformation of GPIIb-IIIa was used (38, 39). A FITC-coupled IgG1 isotype-matched control antibody (Santa Cruz) was used to exclude nonspecific binding. To further confirm GPIIb-IIIa activation, Alexa Fluor 488-labeled human fibrinogen (Invitrogen, Karlsruhe, Germany) was added at a concentration of 100 μg/ml. Flow cytometry was performed within the next hour. In an additional set of experiments, platelets were kept at either 34°C or 31°C for 30 min, followed by their transfer into a 37°C water bath for 30 min and subsequent stimulation by TRAP, as described above.

A FACScan flow cytometer (Becton Dickinson) was calibrated with fluorescent standard microbeads (CalibrITE Beads; Becton Dickinson) for accurate instrument setting. Platelets were identified by their characteristic forward and sideward scatter light and selectively analyzed for their fluorescence properties with the CellQuest program (Becton Dickinson) with assessment of 20,000 events per sample. The relative fluorescence intensity of a given sample was calculated by subtracting the signal obtained when cells were incubated with the...
isotype-specific control antibody from the signal generated by cells incubated with the test antibody.

Whole blood exposure to TRAP/fMLP and flow cytometric analysis of platelet-leukocyte aggregates. Whole blood aliquots of 50 μl were incubated for 25 min with 5 μl of a monoclonal anti-human FITC-coupled CD42b antibody (eBioscience, San Diego, CA) and with 5 μl of a monoclonal anti-human phycoerythrin-coupled CD45 antibody (eBioscience). Aliquots were then incubated at temperatures of 37°C, 34°C, and 31°C in the water bath for 30 min, followed by exposure to TRAP (2.5 mM) and fMLP (10⁻⁷ M) for an additional 30 min. Subsequently, erythrocytes were lysed in 1.5 ml of lysing solution (Becton Dickinson) for 15 min. The reaction was stopped by diluting the solution with 2 ml of PBS, followed by centrifugation at 300 g for 5 min. The aliquots were washed again with PBS, and the pellet was resuspended with 1 ml of Cell Fix. Flow cytometry was performed within the next hour, as described above.

Chemicals. TRAP was purchased from Bachem (Bubendorf, Germany) and dissolved in PBS to yield a 2.5 mM stock solution. fMLP (Sigma-Aldrich, Munich, Germany) was dissolved in PBS and added to the samples to achieve a final concentration of 10⁻⁷ M.

Western blot analysis of tyrosine-specific phosphorylation of platelet proteins. For whole protein extracts and Western blot analysis of let proteins.

Table 1. Blood flow velocity and wall shear rates in mice cremaster muscle microvessels before ferric chloride-induced thrombus formation

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Velocity, μm/s</th>
<th>γ, s⁻¹</th>
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<tr>
<td><strong>Arterioles</strong></td>
<td></td>
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<tr>
<td>37°C</td>
<td>2.235±0.55</td>
<td>172±18</td>
</tr>
<tr>
<td>34°C</td>
<td>1.698±0.159*</td>
<td>181±17</td>
</tr>
<tr>
<td>31°C</td>
<td>1.941±0.222</td>
<td>223±23</td>
</tr>
<tr>
<td><strong>Venules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>1.693±0.185</td>
<td>107±11</td>
</tr>
<tr>
<td>34°C</td>
<td>1.243±0.207</td>
<td>114±18</td>
</tr>
<tr>
<td>31°C</td>
<td>1.864±0.190</td>
<td>70±15</td>
</tr>
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Values are means ± SE; n = 7–9 vessels/group. γ, Wall shear rate. *P < 0.05 vs. 37°C.

Fibrinogen levels and blood cell count. In a separate set of experiments blood was drawn from the retroorbital venous plexus of mice with 37°C, 34°C, and 31°C body temperature for determination of blood cell count and fibrinogen levels (citrate 1:10 vol/vol). The additional use of separate animals was necessary because fluorescent dyes interfere with chemiluminescence reactions. Platelet and red blood cell count was assessed with a cell counter (Sysmex KX-21; Sysmex). Fibrinogen levels were determined by nephelometry (Immage Immunochemistry System; Beckman Coulter, Fullerton, CA) using a polyclonal rabbit anti-human fibrinogen antibody (DAKO Cytomation, Hamburg, Germany).

Statistical analysis. After proving the assumption of normality and equal variance across groups, we assessed differences between groups with one-way ANOVA followed by the appropriate post hoc comparison test. All data were expressed as means ± SE, and overall statistical significance was set at P < 0.05. Pearson product moment correlation was performed to evaluate significant correlations between parameters of platelet activation and temperature. Statistics and graphics were performed with the software packages SigmaStat and SigmaPlot (Jandel, San Rafael, CA).

RESULTS

In vivo thrombosis model. The effect of systemic hypothermia was assessed in vivo by superfusion of microvessels with ferric chloride solution, which resulted in complete thrombotic occlusion of the individually exposed vessel.

At baseline, i.e., before thrombus induction, animals of all groups did not significantly differ with respect to velocity and wall shear rates in arterioles and venules, although hypothermic animals tended to exhibit lower blood flow velocities (Table 1). Quantitative analysis of ferric chloride-induced thrombus formation in controls, i.e., animals maintained at a core body temperature of 37°C, revealed complete occlusion of arterioles and venules after 742 ± 150 and 824 ± 172 s, respectively (Fig. 1). Systemic hypothermia of 34°C caused a significant acceleration in microvascular thrombus formation. Arteriolar and venular vessel lumens were found to be clogged at an average time of 279 ± 120 and 376 ± 121 s, respectively (P < 0.05 vs. 37°C animals). In both arterioles and venules continuous cooling of the animal to a core body temperature of 31°C led to a further decrease in time until complete vessel occlusion occurred (163 ± 57 and 281 ± 71 s, respectively; P < 0.05 vs. 37°C animals).

Within the first 100 and 200 s on ferric chloride superfusion, venular blood cell velocity slowed down by −8% and −20%, respectively, in the control group with 37°C body temperature,
whereas in animals with systemic hypothermia of 34°C, the decrease in venular blood cell velocity was even more pronounced (-37% and -37%, respectively). The deceleration in venular blood cell velocity was found to be abolished at enforced hypothermia of 31°C (-2% and -25%, respectively). Interestingly, hypothermia of 34°C did not decelerate arteriolar blood cell velocity within the first 100 s (-4%) compared with controls of 37°C (-8%) but even caused a

increase of velocities (+14%) when systemic temperature declined to 31°C.

Rewarmed animals exhibited kinetics of thrombus formation comparable to those that were kept continuously at a core temperature of 37°C, although a moderate, but not significant, acceleration in thrombus formation was seen in animals on rewarming from 31°C. Wall shear rates did not differ between the groups. In rewarmed animals, which had been exposed to a 34°C body core temperature for a period of 30 min, arteriolar and venular vessel occlusion occurred at 655 ± 219 and 832 ± 195 s, respectively, and thus was comparable to the kinetics of thrombus formation in animals with a continuous core temperature of 37°C (Fig. 2). In animals that sustained a 30-min cooling period of 31°C, a moderate, but not significant, acceleration in thrombus formation was seen on rewarming to 37°C in both arterioles and venules (453 ± 137 and 469 ± 122 s, respectively).

Flow cytometric analysis of platelet P-selectin expression, GPIIb-IIIa activation, and fibrinogen binding. To closely simulate the clinical situation, we studied moderate degrees of hypothermia and their influences on platelet function in vitro. On incubation at temperatures of 34°C and 31°C, spontaneous expression of P-selectin and the activated conformation of GPIIb-IIIa did not change markedly; however, there was a small but statistically significant increase in PAC-1 binding in unstimulated samples at 31°C, suggestive of spontaneous hypothermia-induced activation (Figs. 3, A and C, and 4). TRAP exposure caused an increase of PAC-1 binding from 1.4 ±

Fig. 2. Occlusion times of arterioles and venules on ferric chloride-induced thrombus formation in animals with normothermia (37°C; n = 6) and animals that underwent systemic hypothermia of 34°C (n = 4) and 31°C (n = 4) for 30 min followed by rewarming up to 37°C. Values are means ± SE.

Fig. 3. Flow cytometric analysis of spontaneous (A and C) and thrombin receptor activating peptide (TRAP)-induced (B and D) expression of platelet P-selectin (A and B) and activation of glycoprotein (GP)IIB-IIIa (C and D) at temperatures of 37°C (solid black line), 34°C (dashed black line), and 31°C (dashed gray line).
0.3% to 68 ± 5% of all platelets, showing the conformational change of GPIIb-IIIa (Fig. 3D). However, the magnitude of activation in response to TRAP increased with decreasing temperatures, as shown by 1.8-fold and 3-fold shifts in mean fluorescence on binding of PAC-1 (Fig. 4B). In line with this, binding of fluorescent-labeled fibrinogen was increased 1.6- and 2.9-fold at 34°C and 31°C after TRAP exposure (P < 0.05 vs. 37°C), whereas only small differences of fibrinogen binding were observed in resting platelets (Fig. 5). TRAP was associated with a marked upregulation of P-selectin expression, as shown by 93 ± 1% of positive platelets (Fig. 3B). Hypothermia caused a small but significant increase of TRAP-induced P-selectin upregulation at 31°C compared with TRAP-stimulated platelets at 37°C (Fig. 4A). Results showed a linear positive correlation between temperature decrease and GPIIIb-IIIa activation, i.e., PAC-1 binding (r = 0.6; P < 0.001), whereas no correlation was found between the temperature decrease and the expression of P-selectin (r = 0.1; P = 0.52).

Platelets maintained at 34°C or 31°C for 30 min and rewarmed to 37°C exhibited spontaneous PAC-1 binding that was indistinguishable from that of platelets kept steadily at normothermic temperatures (Fig. 6). TRAP exposure caused a marked increase of PAC-1 binding on platelets with intermittent hypothermia and rewarmin, which did not differ in magnitude from the response of platelets without hypothermia (Fig. 6). The same results were obtained for spontaneous and TRAP-induced P-selectin expression (data not shown).

Flow cytometric analysis of platelet-leukocyte aggregate formation. Platelet-leukocyte aggregation during hypothermia was determined after stimulation with TRAP/fMLP. Flow cytometric analysis revealed no significant increase after exposure to temperatures of 34°C and 31°C compared with aggregate formation at normothermic temperatures (37°C). After incubation in a 37°C water bath, 14.6 ± 0.9% platelet-leukocyte aggregates were found, represented by the fluorescence intensity of the upper right quadrant in the flow cytometric dot plot. Hypothermia of 34°C and 31°C led to only a marginal, not statistically significant, increase of aggregate formation of 14.7 ± 1.1% and 16.7 ± 1.2%, respectively.

Western blot analysis of tyrosine-specific phosphorylation of platelet proteins. To further underline cold-associated platelet activation, we studied tyrosine-specific protein phosphorylation of platelets at 37°, 34°, and 31°C. As shown in Fig. 7, hypothermia caused enhanced protein phosphorylation at tyrosine residues.
Rosine in resting, but in particular in TRAP-activated, platelets. The most prominent protein bands were found migrating with molecular masses of 130, 125, 95, and 84 kDa (Fig. 7).

**DISCUSSION**

In this report we communicate the following major findings. Moderate systemic hypothermia causes an acceleration of microvascular thrombus formation both in arterioles and venules. These in vivo findings are underlined by in vitro results demonstrating that hypothermia causes a slight increase of spontaneous platelet activation but a marked rise of agonist-induced responsiveness. Although thrombocytic P-selectin seems to play a minor role in cold-enhanced cell-cell contact, the fibrinogen receptor GPIIb-IIIa is influenced in a major way by temperature changes. The present results provide in vivo evidence for the frequently observed coincidence of increased rates of thrombotic events at reduced core temperatures.

During cold temperatures, impaired rheological properties of the blood, namely, reduced viscosity (21) as well as enhanced stiffness and reduced deformability of cells (3, 26), may compromise tissue perfusion. In addition, a reduction in temperature results in an increase in platelet and red blood cell numbers as well as a reduction in plasma volume (18, 27), further impeding capillary passage and thus nutritive blood flow. These changes together with the winter rise in fibrinogen concentrations (44) have all been used as probable explanations for rapid increases in coronary and cerebral thrombosis in cold weather (18, 30).

In the present study no significant differences in blood cell count were observed at clinically occurring moderate hypothermia of 34°C and 31°C compared with normothermia. However, blood fibrinogen levels were found to be significantly reduced at hypothermic temperatures, most likely because of an increase of consumption on microvascular thrombus formation. Many clinical studies of the past revealed a bleeding diathesis rather than a prothrombotic state in hypothermic individuals (7, 19, 28, 31, 36). Given the fact that a decrease of fibrinogen levels and an increase in microvascular thrombus formation were observed in the present study at core temperatures of 34°C and 31°C, it might be hypothesized that hypothermic temperatures lead to a rise in microvascular thrombus formation via activation of the GPIIb-IIIa receptor and subsequent fibrinogen binding, possibly resulting in coagulopathy later on in the process.

Increased reactivity and adhesiveness of cells may lead to adherence to the vascular lining with partial obturation of vascular cross sections and propagation of complete microvascular blockage. In particular, on exposure of the vessel to a noxious stimulus such as ferric chloride (6, 22), local oxidant stress-induced endothelial injury provides a preferential site for
cell trapping with thrombus growth. Although the importance of GPIb-IX-V in mediating platelet-endothelial interactions is unequivocal, this ligand seems mandatory for adhesion and thrombus growth at high shear (4). On the contrary, at low shear other adhesion molecules, such as the collagen receptors and GPIb-IIIa, are mainly involved (4, 25, 33). The vessels monitored in the present study presented with wall shear rates below 300 s⁻¹. Thus we preferentially studied platelets and their cold-related change in activation of the fibrinogen receptor GPIb-IIIa. It has been shown that unstimulated platelets attach to fibrinogen in a selective, GPIb-IIIa-dependent process and that the initial attachment is followed by spreading and irreversible adhesion, even in the absence of exogenous agonists or the presence of activation inhibitors (35). In addition, the specific synergy of multiple substrate-receptor interactions with coupling of functions of GPIb with GPIb-IIIa, allowing the latter to arrest platelets on von Willebrand factor under conditions not permissive for direct binding to fibrinogen, underscores the crucial role of GPIb-IIIa for thrombogenesis (34). Past studies have shown that thrombin-induced tyrosine phosphorylation of several proteins is dependent on platelet aggregation mediated by fibrinogen binding to GPIb-IIIa (11, 15). The present results, demonstrating the massive PAC-1 binding of platelets with reduced temperature, indicative of the activated conformation of GPIb-IIIa (39), underscore the importance of this adhesive receptor in cold-related pathology. Moreover, fibrinogen binding to TRAP-activated platelets and tyrosine-specific phosphorylation of platelet proteins were increased at 34° and 31°C, further substantiating the activating effect of cold on the GPIb-IIIa receptor.

The present observation that TRAP barely started to increase P-selectin expression at a temperature of 31°C goes along with results of Faraday and Rosenfeld (10), reporting only a modest (1.6 fold) TRAP-induced increase of platelet P-selectin expression at 22°C vs. 37°C compared with an almost 25-fold higher rise in PAC-1 binding. This might imply that P-selectin expression is less temperature sensitive than GPIb-IIIa, although the effects may in addition greatly depend on the agonists used and the time point of analysis. For example, hypothermia-induced reduction of P-selectin expression, as observed by Michelson et al. (24), was only transient in nature and absent within 10 min after agonist exposure. Because hypothermia failed to influence platelet-leukocyte aggregate formation and because P-selectin is believed to be a key player in this cellular cross talk (14), the present results further emphasize the inferior role of platelet P-selectin expression in mediating hypothermia-associated thrombosis.

Our in vitro data confirm those of others, reporting that hypothermia induces platelet activation in vitro, as indicated by changes in platelet shape and morphology (23, 43), tyrosine-specific protein phosphorylation (11), and fibrinogen receptor exposure and activation as well as platelet aggregation (29). However, we significantly extend current knowledge in that we could prove the relevance of systemic hypothermia in the enhancement of microvascular thrombus formation in vivo. Hypothermic temperatures above 30°C were purposely chosen because they are frequently encountered in a number of clinical settings, such as major surgery, multiple trauma, cold exposure, and the neonatal period (5, 16, 31, 41). It has been shown that unintentional hypothermia is associated with myocardial ischemia, angina, and impaired ventilation and blood oxygenation during the early postoperative period in patients undergoing lower-extremity vascular surgery (12). The impact of temperature is further underscored by the fact that the simple perioperative maintenance of normothermia in patients with cardiac risk factors was associated with a reduced incidence of morbid cardiac events and ventricular tachycardia (13). Thus the enhanced risk for microvascular thrombus formation during cold is best counteracted by an immediate rewarming of the patient, as shown by the complete reversion of platelet hyperresponsiveness on reexposure to 37°C. In line with this, animals subjected to hypothermia followed by rewarming have not been found to be prone to enhanced thrombogenesis.

In summary, moderate systemic hypothermia accelerates microvascular thrombosis, which might be mediated by increased GPIb-IIIa activation on platelets, but does not cause predisposition with increased risk for microvascular thrombus formation after rewarming. Thus maintaining normothermia or rewarming hypothermic patients represents a common goal in limiting the risk for cold-associated thrombotic events.

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