Critical temperature ranges of hypothermia-induced platelet activation: Possible implications for cooling patients in cardiac surgery

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Summary

Cooling of the patient is routinely applied in cardiac surgery to protect organs against ischemia. Hypothermia induces activation of platelets, but the effects of temperatures such as used during cardiac surgery are not well described. To investigate this in an in-vitro study heparinized whole blood was incubated at different temperatures (37°C, 34.5°C, 32°C, 29.5°C, 27°C, 24.5°C, 22°C, 19.5°C and 17°C). The effect of these temperatures on aggregation, P-selectin expression, GP IIb/IIIa activation and platelet microparticle (PMP) formation of unstimulated and ADP-stimulated platelets of 36 subjects was evaluated in flow cytometry. A four-parametric logistic model was fitted to depict the temperature effect on platelet parameters. Lower temperatures increased aggregates, P-selectin expression, and GP IIb/IIIa activation. The number of PMPs decreases with hypothermia. Additional experiments revealed a slight influence of heparin on platelet P-selectin expression but excluded an effect of this anticoagulant on the other evaluated parameters. Threshold temperatures, which mark 5% changes of platelet parameters compared to values at 37°C, were calculated. On ADP-stimulated platelets the thresholds for P-selectin expression and GP IIb/IIIa activation are 34.0°C and 36.4°C, respectively, and lie in the temperature range routinely applied in cardiac surgery. Hypothermia-induced platelet activation may develop in most patients undergoing cardiac surgery, possibly resulting in thromboembolic events, coagulation defects, and proinflammatory leukocyte bridging by P-selectin bearing platelets and PMPs. These findings suggest that pharmacological protection of platelets against hypothermia-induced damage may be beneficial during cardiac surgery.

Keywords

Platelet physiology, cell-cell interactions, surgery, platelet glycoproteins

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Introduction

Hypothermia is routinely used in cardiac surgery during cardiopulmonary bypass (CPB) and especially for deep hypothermic circulatory arrest (DHCA) to protect organs against ischemia. Applied temperatures include so-called "mild" (31–34°C), "moderate" (25–30°C), and "deep hypothermia" (< 20°C) (1). In connection with platelet preservation and from in-vitro studies it has been reported that hypothermia in the range of 4°C to 32°C induces platelet-aggregate formation by an unknown mechanism. Temperatures in the range of deep hypothermia have been reported to induce activation of the platelet fibrinogen receptor GP IIb/IIIa as well as expression of P-selectin, which mediates proinflammatory platelet-leukocyte binding (2–5). Another possible response of platelets to activation is the release of microparticles from the plasma membrane into the circulation. Platelet microparticle (PMP) formation has been reported to occur during cardiopulmonary bypass (CPB) (6, 7). PMPs exert a strong procoagulant activity by formation of a prothrombinase complex on their surface and bear typical platelet surface receptors including P-selectin (7–9).

Hypothermia-induced platelet aggregates have been postulated to cause microthrombembolism followed by cognitive dysfunction in patients undergoing hypothermic open-heart surgery

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(2). Furthermore, a hypothermia-related loss of platelet function may result in bleeding complications during cardiac operations. Nevertheless, neither the temperature at which platelets begin to be influenced by hypothermia, nor the effects of mild and moderate hypothermia, as routinely used in cardiac surgery, have been clearly stated.

The goal of this study was to evaluate the effect of mild, moderate and deep hypothermia on platelet function and to find the temperature range at which the formation of platelet aggregates and PMPs, as well as GP IIb/IIIa activation and P-selectin expression begin to be influenced.

Materials and methods

Experiments on the effect of gradual temperature decreases on platelet function in heparinized blood as depicted in Figure 2A-E were performed in the research laboratory facilities of the Department of Thoracic, Cardiac and Vascular Surgery of the University of Tübingen, Germany. Experiments and blood sampling procedures were approved by the local ethics committee of the University of Tübingen, Germany. Blood was sampled from 36 subjects (8 subjects were female and 28 subjects were male; ethnical background: all caucasian). All subjects were older than 18 years and were mainly recruited amongst medical students with an average age range of 20 to 25 years. The oldest subject was a 49-year-old male. All subjects gave written informed consent for their participation in the study. To compare possible activating effects of the anticoagulants heparin and r-hirudin in combination with stimulation using ADP, collagen or convulxin on platelets experiments were performed at the Baker Heart Research Institute (Centre for Thrombosis & Myocardial Infarction; Melbourne, Australia) with blood from six subjects (1 subject was female and 5 subjects were male; ethnical background: 5 caucasian, 1 asian; age range: 28–46 years old). Blood sampling and incubation procedures were performed according to a previously described method (5).

Blood sampling

Blood of non-medicated healthy subjects free of platelet-affecting drugs for 14 days was collected by venous puncture. The first 5 ml were discarded before blood was drawn for analysis. Blood was anticoagulated with 3 IU/ml heparin or 20 µg/ml r-hirudin, divided into different samples and processed immediately.

Sample preparation to evaluate effects of gradual temperature decreases on platelets

Nine sets of heparinized blood samples were used for testing nine different temperatures. Each set consisted of two subsets of blood samples: The first subset consisted of two samples each containing 25 µl of heparinized whole blood that was incubated with an antibody combination of 2.5 µl anti-CD41-FITC (SZ 22, Beckman-Coulter GmbH, Krefeld, Germany) and 2.5 µl anti-CD62-PE (BD Biosciences, Heidelberg, Germany) and served for evaluation of platelet aggregation, PMP formation and P-selectin expression. The second subset contained 25 µl of a dilution (1:50) of heparinized whole blood in modified Tyrode´s buffer and was incubated with 5 µl of the PAC-1-FITC antibody (BD Biosciences) that specifically binds to the activated GP IIb/IIIa receptor. One sample of each subset was treated with 5 µl of the platelet activator ADP (Mölab, Hilden, Germany; final concentration: 10 µM), the remaining sample was treated with 5 µl of phosphate-buffered saline (PBS)-buffer to achieve equal volumes.

Temperature conditions

The blood sample sets of each subject were incubated in parallel for 30 minutes (min) at different temperatures (37°C, 34.5°C, 32°C, 29.5°C, 27°C, 24.5°C, 22°C, 19.5°C and 17°C) using a specially designed "temperature-regulation" device that consists of nine incubation blocks each with a heating and cooling function. Each incubation block was heated (or cooled) to one of the above mentioned temperatures and has apertures to carry the four samples of a set. The sample tubes in the incubation blocks were preloaded with antibodies and ADP-solution or PBS buffer according to the sample preparation protocol. Afterwards blood was added so that the complete sample was immediately incubated at the designated temperature. After an incubation period of 30 min all samples were fixed with CellFix® (BD Biosciences).

Sample preparation to evaluate effects of heparin and hirudin and of different agonists on platelets

Four sets of blood samples, two of these anticoagulated with heparin and two anticoagulated with hirudin, were prepared. Each set consisted of two subsets of blood samples: The first subset contained four samples with 25 µl of whole blood. Each of these samples was incubated with an antibody combination of 2.5 µl anti-CD41-FITC (SZ 22, Beckman-Coulter GmbH, Krefeld, Germany) and 2.5 µl anti-CD62-PE (BD Biosciences, Heidelberg, Germany) to evaluate platelet aggregation, PMP formation and P-selectin expression. The second subset contained a pair of samples with 25 µl of a dilution (1:50) of whole blood in modified Tyrode´s buffer that was incubated with 5 µl of the PAC-1-FITC antibody (BD Biosciences). In the first subset one sample was treated with 5 µl of the platelet activator ADP (Mölab, Hilden, Germany; final concentration: 10 µM), the second sample with collagen (Kollagenreagens Horm, Nycomed Austria GmbH, Linz, Austria; final concentration: 20 µg/ml), the third sample with convulxin (Pentapharm, Basel, Switzerland; final concentration: 20 ng/ml) and a control sample with 5 µl of PBS-buffer to achieve equal volumes. In the second subset one sample of each pair was treated with 5 µl of the platelet activator ADP (Mölab, Hilden, Germany; final concentration: 10 µM), and the remaining sample was treated with 5 µl of PBS-buffer to achieve equal volumes. Of the four initial sample sets, two sets (one heparinized and one hirudinized) were incubated for 30 min at 37°C, and the other two sets (one heparinized and one hirudinized) were incubated in parallel at 17°C.

Flow cytometry

Flow cytometry was performed within 6 hours (h) after sample fixation on a FACScan® cytometer (Becton Dickinson, Heidelberg, Germany) at the University of Tübingen, Germany (evaluation of effects of gradual temperature decreases on platelets) and on a FACS Calibur® cytometer at the Baker Heart Research Institute in Melbourne, Australia (evaluation of effects of heparin, hirudin and of different agonists on platelets). Before start of experiments, the flow cytometer in Tübingen was calibrated with latex beads (Polysciences, Eppelheim, Germany) of defined

sizes. According to measurements with 1- and 3-µm-sized beads three regions were set in a forward scatter (FSC) / sideward scatter (SSC) dot plot to distinguish between platelet aggregates, single platelets and PMPs, regarding their FSC (size) properties. Represenative dot plots that depict the response of platelets after stimulation with different agonists are given in Figure 1. According to calibration with 1-µm-sized beads PMPs are defined in this study as CD41-positive objects with a size of ≤1 µm. The amplification settings of the FSC-axis of the FACScan (University of Tübingen) and FACScalibure (Baker Heart Research Institute, Melbourne) were identical (E00 / log 1.0). For the experiments performed in Melbourne the borders of the PMP, single platelet and aggregate regions were confirmed using polystyrene calibration particles (Spherotech Inc., Lake Forest, IL, USA).

The following measurements were performed with samples of the first subset (anti-CD41/62 antibody combination): Samples were evaluated according to a method described by Nieuwland et al. (7) with some modifications. Samples were analyzed by triggering on a preset threshold of the platelet marker SZ 22-FITC fluorescence. Objects positive for SZ 22-binding were analyzed. Amounts of platelet aggregates and PMPs were calculated and are given as percentages of counts in their particular region per 10,000 total events that were counted in each measurement. Furthermore, anti-CD62-PE fluorescence was analyzed in each region for samples of the first subset. In samples of the second subset in each temperature group single platelets were detected regarding their forward and sideward scatter characteristics according to another previously described method (5). The percentage of platelets positive for PAC-1 binding as a measure for GP IIb/IIIa activation was analyzed.

Statistics

Evaluation of the effect of gradual temperature decreases on platelets

For each of the five variables [percentages of (i) platelet aggregates and (ii) PMPs; P-selectin expression on (iii) aggregate-bound platelets and (iv) PMPs; (v) PAC-1 binding] with and without ADP-stimulation we fitted a four-parametric logistic model to the observations for each of the 36 subjects separately. For 63 out of these 360 curves the slope parameter c turned out to be 0. These 63 curves were excluded from further analysis. Altogether there were 2,673 values (=297 curves x 9 temperatures). Out of those, 49 values (i.e. 1.8%) were excluded as outliers. For each variable with and without ADP-stimulation and each temperature we calculated the expected values by a logistic curve using the mean parameters of all subjects and compared these expected values with the 95% confidence limits of the observed means.

In the fitted curves that are shown in Figure 2A-E the relationship of the depicted variables to the incubation temperature is calculated by the equation $y = a + (b-a) / [1+e^{-c(T-T50)}]$, in which

- a is the initial value reached at low temperatures
- b is the final value reached at high temperatures
- T_{50} is the temperature at which the mean value between a and b is reached
- c is the parameter that determines the slope of the curve at the inflection point T_{50}.

The goodness of fit of the model was assessed by an overall coefficient of determination of 99% and a root mean squared error of 2.7%. The model was established similar to a method described by J. K. Lindsey (10).

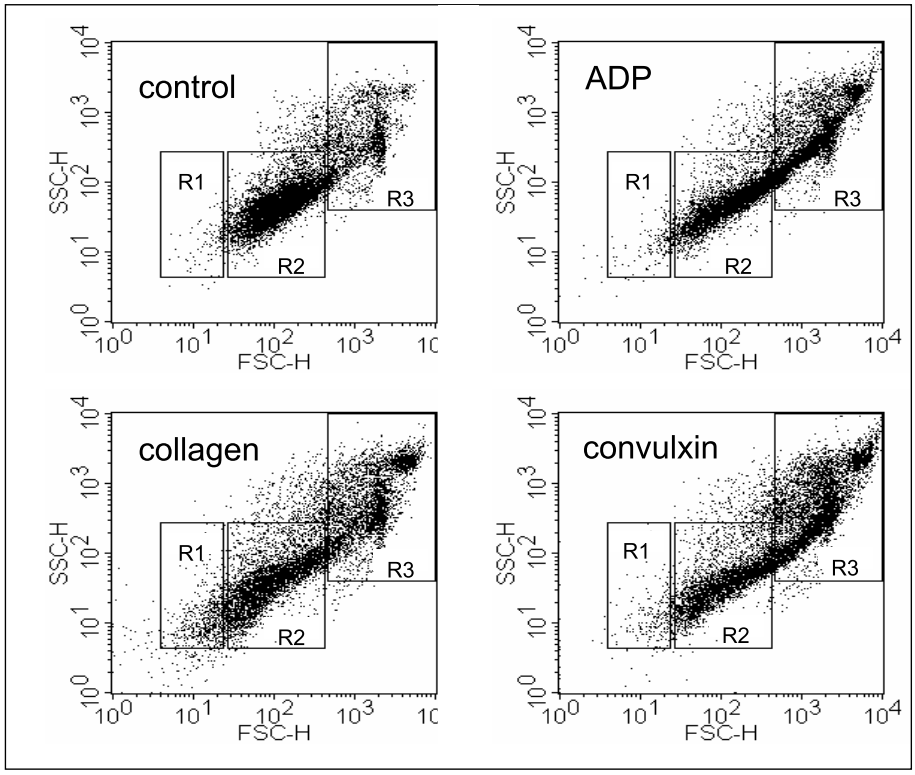

Figure 1: Four dot-plots that are representative for measurements performed in this study are given to depict the effect of stimulation with 10 µM ADP, 20 µg/ml collagen and 20 ng/ml convulxin on the formation of platelet microparticles (detected in region "R1"), single platelets (detected in region "R2") and platelet aggregates (depicted in region "R3"). The depicted measurements were obtained from samples that were incubated at 37°C for 30 minutes. Regions were set according to measurements with calibration beads of defined sizes. Compared to control there is a marked increase of the density of platelet microparticles upon stimulation with collagen as well as convulxin and of platelet aggregates upon stimulation with all agonists.

From the fitted curves we calculated the temperature at which the minimum or maximum percentage was either increased or decreased by 5% in comparison to the percentages observed at 37°C for each variable. This temperature is defined as "threshold temperature". To depict the effect of ADP-stimulation, the differences of mean values between ADP-stimulated and unstimulated samples for a, b, c and T_{50} were calculated. A two-sided paired t-test was used to evaluate differences between unstimulated and ADP-stimulated samples of each variable (see Table 1). These analyses were performed using JMP statistical software (versions 5.0 and 5.1).

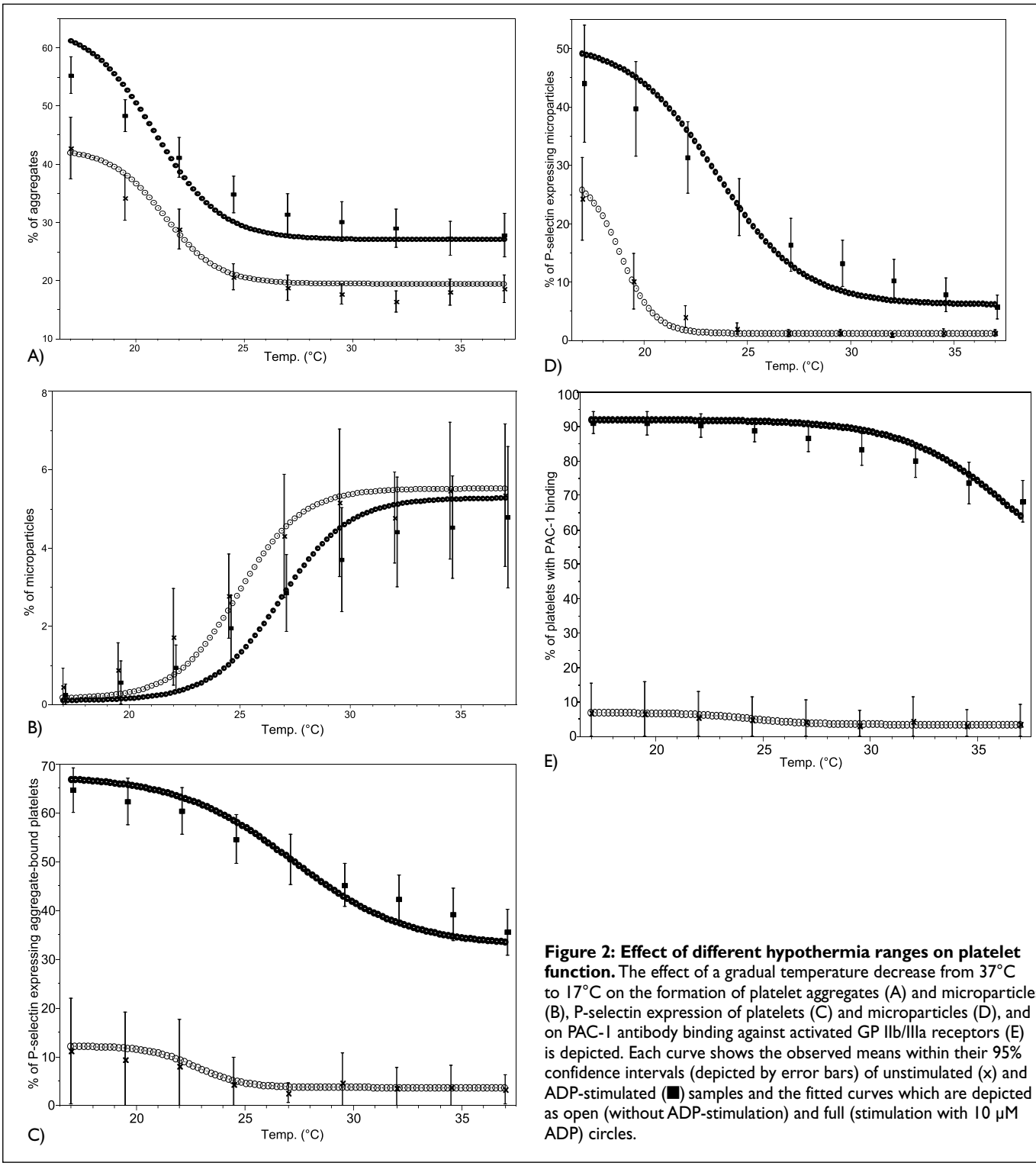

Figure 2: Effect of different hypothermia ranges on platelet function. The effect of a gradual temperature decrease from 37°C to 17°C on the formation of platelet aggregates (A) and microparticles (B), P-selectin expression of platelets (C) and microparticles (D), and on PAC-1 antibody binding against activated GP IIb/IIIa receptors (E) is depicted. Each curve shows the observed means within their 95% confidence intervals (depicted by error bars) of unstimulated (x) and ADP-stimulated (■) samples and the fitted curves which are depicted as open (without ADP-stimulation) and full (stimulation with 10 µM ADP) circles.

		Mean difference between ADP-stimulated and -unstimulated samples (%)	95% CI of mean difference (%)	P-value
Aggregates	37°C	8.72	6.3 – 11.2	<0.0001
	17°C	14.1	8.0 – 20.2	<0.0001
	T_{50}	0.54	–0.5 – 1.6	0.2914
	Slope	–0.22	–0.4 – –0.006	0.0441
P-sel-plt	37°C	33.23	22.48 – 43.99	<0.0001
	17°C	63.94	53.16 – 74.72	<0.0001
	T_{50}	6.05	3.47 – 8.63	0.0005
	Slope	–0.57	–0.80 – –0.33	0.0004
PAC-1	37°C	31.59	13.82 – 49.36	0.0015
	17°C	83.89	74.22 – 93.56	<0.0001
	T_{50}	12.77	8.06 – 17.49	<0.0001
	Slope	–0.35	–0.52 – –0.18	0.0005
PMP	37°C	–0.25	–0.86 – 0.37	0.4294
	17°C	–0.05	–0.19 – 0.08	0.4184
	T_{50}	1.88	0.78 – 2.98	0.0014
	Slope	–0.06	–0.19 – 0.08	0.3767
P-sel-PMP	37°C	5.14	3.19 – 7.1	<0.0001
	17°C	21.87	11.30 – 32.43	0.0003
	T_{50}	3.93	2.64 – 5.22	<0.0001
	Slope	-0.57	–0.75 – –0.39	<0.0001

Table 1: Effect of sample stimulation with ADP (10 μM) on platelet aggregate formation (aggregates), P-selectin expression of platelets (P-sel-plt), PAC-1 antibody binding (PAC-1), platelet microparticle generation (PMP), and P-selectin expression on platelet microparticles (P-sel-PMP). Mean differences and their 95% confidence intervals (CI) between ADP-stimulated and unstimulated samples and p-values of a two-sided paired t-test, which was used to analyze the significance of the mean differences, are given for each parameter at 37°C and 17°C, for T_{50} (in °C) and for the slope parameter at T_{50}. Mean differences and their 95% CI are given in percent.

Evaluation of the effects of heparin, hirudin and different stimulants

For all values the logit-transformation was adopted to stabilize the variances. In order to perform the logit transformation for all data, two values of P-selectin expression on microparticles that had been measured as 0% were replaced by 1% and one value of the same variable that had been measured as 100% was replaced by 99%. For each of the five variables [percentages of (1) platelet aggregates and (2) PMPs; P-selectin expression on (3) aggregate-bound platelets and (4) PMPs; (5) PAC-1 binding] we performed a multifactorial analysis of variance to evaluate the effects of the different temperatures, anticoagulation methods and stimulants. The individual subject was included into the models as random factor. The Tukeys HSD test with a global alpha of 5% was used to compare the effect of different stimulants on the amount of PMP formation. After the fitting of the model the observed and predicted means were backtransformed and are given together with their 95-% confidence intervals in Tables 2a and 2b. These analyses were performed using JMP statistical software (version 6.0.3).

Results

Platelet aggregation in response to a temperature decrease begins at moderate hypothermia (Fig. 2A)

In both unstimulated and ADP-stimulated samples, induction of platelet aggregate formation is not observable at mild hypothermia; it starts at temperatures in the range of moderate hypothermia, and is maximal at deep hypothermia. The threshold temperatures at which aggregates are induced by hypothermia by 5% were calculated as 25.2°C for unstimulated and 25.8°C for ADP-stimulated samples.

PMP formation is decreased with hypothermia (Fig. 2B)

In both unstimulated and ADP-stimulated samples, PMP formation is decreased by hypothermia and reaches a minimum in the range of deep hypothermia. The threshold temperatures for the decrease of PMPs are 29.1°C for unstimulated and 31.3°C for ADP-stimulated samples.

Hypothermia-induced P-selectin expression and GP IIb/IIIa activation: Distinct differences between unstimulated and ADP-stimulated samples (Fig. 2C-E)

Considerable differences of P-selectin expression and PAC-1 binding in response to hypothermia are observed between unstimulated and ADP-stimulated samples.

In unstimulated samples no induction of P-selectin expression is observed at mild hypothermia on platelets. A further decrease of the incubation temperature into the range of moderate hypothermia initiates P-selectin expression on platelets that becomes maximal at deep hypothermia. In ADP-stimulated samples a gradual increase of P-selectin is observed that starts in the range of mild hypothermia and reaches a maximum at deep hypothermia.

Table 2: Effect of the anticoagulants heparin and hirudin and the platelet agonists ADP, collagen and convulxin on platelet aggregation ("aggregates") and microparticle formation ("platelet microparticles") (a) and on the P-selectin expression of platelets (P-sel-plt) and microparticles (P-sel-PMP) as well as PAC-1 binding ("PAC-1") at 17°C and 37°C (b). Data are given as backtransformed observed and predicted means together with their 95% confidence intervals (n=6).

a)

Temp. [°]	Agonist	Anticoagulation	Aggregates			Platelet microparticles		
			Obs. mean	Pred. mean	95%-CI	Obs. mean	Pred. mean	95%-CI
17	Control	Heparin	39.4	36	27.6 - 45.2	0.33	0.33	0.19 - 0.58
		Hirudin	37.2	36	27.6 - 45.2	0.51	0.33	0.19 - 0.58
	ADP	Heparin	62.7	60.1	50.6 - 68.9	0.37	0.29	0.16 - 0.5
		Hirudin	62.2	60.1	50.6 - 68.9	0.29	0.29	0.16 - 0.5
	Collagen	Heparin	49.6	54.5	44.9 - 63.8	0.57	0.53	0.3 - 0.93
		Hirudin	56.8	54.5	44.9 - 63.8	0.32	0.53	0.3 - 0.93
	Convulxin	Heparin	59.3	65.8	56.7 - 73.9	0.59	0.52	0.3 - 0.91
		Hirudin	65.4	65.8	56.7 - 73.9	0.37	0.52	0.3 - 0.91
37	Control	Heparin	17.8	18.6	13.5 - 25.2	1.42	1.8	1.04 - 3.13
		Hirudin	16.6	18.6	13.5 - 25.2	1.51	1.8	1.04 - 3.13
	ADP	Heparin	43	38	29.4 - 47.5	1.38	1.57	0.9 - 2.72
		Hirudin	29.1	38	29.4 - 47.5	1.38	1.57	0.9 - 2.72
	Collagen	Heparin	33.3	32.9	25 - 41.9	3.46	2.87	1.65 - 4.92
		Hirudin	34.8	32.9	25 - 41.9	3.66	2.87	1.65 - 4.92
	Convulxin	Heparin	51	44	34.8 - 53.6	3.12	2.83	1.63 - 4.86
		Hirudin	44.3	44	34.8 - 53.6	3.23	2.83	1.63 - 4.86

b)

Temp. [°]	Agonist	Anticoagulation	P-sel-plt			P-sel-PMP			PAC-1		
			Obs. mean	Pred. mean	95%-CI	Obs. mean	Pred. mean	95%-CI	Obs. mean	Pred. mean	95%-CI
17	Control	Heparin	9.5	9.6	6.3 - 14.4	5	5.5	3.1 - 9.6	3.3	2.5	1.7 - 3.7
		Hirudin	6.8	6.7	4.3 - 10.2	6	5.5	3.1 - 9.6	2.2	2.5	1.7 - 3.7
	ADP	Heparin	77	75.7	66.2 - 83.1	29.7	29.7	18.7 - 43.6	87.9	89	84.4 - 92.3
		Hirudin	66	67.7	57 - 76.9	29.7	29.7	18.7 - 43.6	88.6	89	84.4 - 92.3
	Collagen	Heparin	41	38.4	28.2 - 49.7	30.3	30.3	19.2 - 44.4			
		Hirudin	27.4	29.6	21 - 40	30.4	30.3	19.2 - 44.4			
	Convulxin	Heparin	96.8	95.7	93.4 - 97.3	65.9	74.4	61.3 - 84.2			
		Hirudin	91.8	93.8	90.5 - 96	81.3	74.4	61.3 - 84.2			
37	Control	Heparin	6.3	8.5	5.5 - 12.8	6.2	6.2	3.5 - 10.8	0.6	0.7	0.5 - 1.1
		Hirudin	7.9	5.9	3.8 - 9	6.2	6.2	3.5 - 10.8	0.7	0.7	0.5 - 1.1
	ADP	Heparin	67	62.7	51.5 - 72.8	21.9	16.6	9.8 - 26.7	71.8	69.4	60.4 - 77.2
		Hirudin	48.6	53.2	41.8 - 64.3	12.4	16.6	9.8 - 26.7	70	69.4	60.4 - 77.2
	Collagen	Heparin	57.9	66.1	55.1 - 75.5	25.3	25.9	16 - 39.1			
		Hirudin	65	56.8	45.3 - 67.6	26.6	25.9	16 - 39.1			
	Convulxin	Heparin	89.3	89.3	84.1 - 93	39	34.4	22.2 - 49			
		Hirudin	85	85	78.1 - 90	30	34.4	22.2 - 49			

On PMPs P-selectin is unchanged at mild and moderate hypothermia in unstimulated samples. At deep hypothermia a strong increase of P-selectin expression is observed. In ADP-stimulated samples a gradual increase of P-selectin is observed that starts in the range of mild hypothermia and reaches a maximum at deep hypothermia. The threshold temperatures for P-selectin expression are 26.9°C for platelets and 23.9°C for PMP in unstimulated samples as well as 34.0°C for platelets and 33.4°C for PMP in ADP-stimulated samples.

PAC-1 antibody binding, which indicates GP IIb/IIIa activation, is not influenced by mild hypothermia in unstimulated samples. A temperature decline to moderate hypothermia leads to a slight increase of GP IIb/IIIa activation with a maximum at deep hypothermia. In ADP-stimulated samples a gradual increase of GP IIb/IIIa activation is observed that starts in the range of mild hypothermia. The threshold temperatures for PAC-1 antibody binding are 28.7°C for unstimulated and 36.4°C for ADP-stimulated samples.

Effect of ADP stimulation on curve parameters

The effects of sample stimulation with 10 μM ADP on the curve parameters a, b, T_{50} and c of each variable are given in Table 1.

Effects of heparin, hirudin and different stimulants at 17°C and 37°C

Formation of platelet aggregates and microparticles (Fig. 1 and Table 2a)

Neither platelet aggregation nor PMP formation are significantly influenced by anticoagulation with heparin or hirudin. Platelet aggregates increase according to stimulation with the different agonists and with hypothermia.

PMP formation is significantly influenced by stimulation with different agonists ($p=0.0071$) and by temperature ($p<0.0001$): At 37°C stimulation with collagen (2.5-fold increase in heparinized and 2.7-fold increase in hirudinized samples) or convulxin (2.3-fold increase in heparinized samples and 2.3-fold increase in hirudinized samples) significantly enhances the number of PMPs when compared to stimulation with ADP. At 17°C less PMPs are generally present, but PMP numbers also increase with the addition of collagen (1.5-fold in heparinized and 1.1-fold in hirudinized samples) or convulxin (1.6-fold in heparinized samples and 1.3-fold in hirudinized samples) in comparison to ADP-stimulated samples.

P-selectin expression and GP IIb/IIIa activation (Table 2b)

P-selectin expression on platelets is significantly influenced by different anticoagulants ($p=0.0021$) and by the interaction of stimulation with temperature ($p<0.0001$): When comparing heparinized to hirudinized samples, six samples showed higher P-selectin expression with heparin compared to hirudin (at 17°C: 1.4-fold increase in controls, 1.2-fold increase with ADP, 1.5-fold increase with collagen, and 1.1-fold increase with convulxin; at 37°C: 1.4-fold increase with ADP and 1.1-fold increase with convulxin) and two samples showed higher P-selectin expression with hirudin compared to heparin (at 37°C: 1.3-fold increase in controls and 1.1-fold increase with collagen). On PMPs, P-selectin expression is also significantly influenced by the interaction of stimulation with temperature ($p=0.0011$; higher values caused by hypothermia and stimulants) but not by the different anticoagulation methods.

Binding of the GP IIb/IIIa activation-specific antibody PAC-1 is not influenced by different anticoagulation methods but by temperature ($p<0.0001$) and stimulation ($p<0.0001$) with higher values caused by hypothermia and the different agonists.

Discussion

Our experiments give an insight into the effects of a gradual temperature decrease from normo- to hypothermia on platelet function. Differences in the temperatures at which P-selectin expression, GP IIb/IIIa activation and aggregation occur were observed. The threshold temperature which effects P-selectin expression and GP IIb/IIIa activation is higher than the temperature which leads to platelet aggregate formation. These findings suggest that a distinct level of hypothermia-induced platelet activation is required and a certain number of GP IIb/IIIa receptors need to be activated before hypothermia-induced platelet aggregates are formed. Furthermore, threshold temperatures for P-selectin expression, GP IIb/IIIa activation and platelet aggregation are higher in ADP-stimulated than in unstimulated samples. Therefore, hypothermia not only acts as an autonomous platelet activator, but also enhances platelet responses to stimulation with ADP, thereby serving as a cofactor for platelet activation.

Our study shows that in contrast to hypothermia-induced increases of typical platelet activation markers, the shedding of PMPs, which is also a possible response of platelets to activation, is inhibited by hypothermia. The exact mechanism of PMP generation has not been elucidated yet. However, it has been reported that their formation may result from hydrolysis of actin-membrane interactions within the platelet membrane skeleton by the protease calpain. Thereby, local associations of the platelet membrane skeleton with the plasma membrane are disrupted, followed by microparticle shedding (11). Hypothermia has been reported to induce phase changes of platelet membrane lipids (12), and membrane phase changes seem to uncap actin filaments, resulting in actin assembly-mediated alterations of platelet shape (13). These facts indicate that the decrease of PMP formation by hypothermia may be explained by hypothermia-induced alterations of processes that regulate platelet membrane and cytoskeleton functions. Indeed it has been shown that aggregate formation is directly dependent on the interaction between cytoskeleton and the integrin GP IIb/IIIa (14). P-selectin, which is expressed on PMPs, has been reported to bridge leukocytes. PMPs may thereby enhance leukocyte accumulation by promoting the tethering of flowing leukocytes to leukocytes that are already rolling on the endothelium. As a result PMPs may not only augment hemostasis but may even amplify leukocyte-mediated tissue injury in thrombotic and inflammatory disorders (9). ADP-stimulation had no effect on PMP formation at low and high temperatures and on the curve slope at T_{50} in our experiments, which is in concordance with other studies that also reported a missing effect of ADP on PMP formation (15). However, our experiments show a significant influence of ADP on the T_{50} of PMP formation, which is higher in ADP-stimulated samples. This finding indicates that especially in the range of moderate hypothermia ADP seems to decrease microparticle generation. Because the experiments per-

formed in this study are limited on the evaluation of platelet surface receptors, an insight into the underlying mechanism of this finding cannot be given. Possibly this phenomenon may be explained by modulations of the platelet cytoskeleton by hypothermia in combination with ADP.

To compare PMP formation in ADP-stimulated samples with other agonists, the effect of the platelet stimulants collagen and convulxin, which have been reported to be potent inductors of PMP formation (15), were investigated. In contrast to ADP both collagen and convulxin significantly increased PMP formation, which is in concordance with the literature (15). The stimulating effect of collagen and convulxin on PMP formation is more pronounced at 37°C but the tendency of increased PMP formation by collagen and convulxin can be seen at both temperatures.

Despite the decrease of PMPs with hypothermia, the expression of P-selectin on PMPs increases strongly. Therefore, the fraction of PMPs that is formed during hypothermia may influence leukocyte function, induce inflammatory events, and carry a significant procoagulatory potential. Procoagulant PMPs are generated during coronary bypass surgery and are present in systemic and pericardial blood (7). This finding indicates the importance of PMP formation during cardiac surgery that may be modulated by hypothermia.

Contact of patient blood with the artificial surfaces of the extracorporeal circulation circuit induces a complex activation of the coagulation system that is accompanied by a release of platelet activators (16). Shear stress which occurs during extracorporeal circulation results in ADP-release of platelets and erythrocytes with subsequent platelet activation and aggregation (17, 18). In most cardiac operations employing cardiopulmonary bypass, mild, moderate or deep hypothermia is used for organ protection against ischemia. Our experiments support that, in addition to circulation within the ECC-circuit, the routine application of hypothermia may have an impact on platelet function during and after cardiac surgery. Platelets may become activated by hypothermia, and their reactions to ADP and other activators may be amplified by hypothermia. As a consequence of hypothermia-induced activation, platelets may be consumed in aggregates and loose their function. This may result in platelet-related bleeding complications, thromboembolism, and platelet- and PMP-mediated inflammatory reactions in patients operated on in hypothermia.

It has been reported that heparin induces platelet P-selectin expression and the activation ability of platelets (19). Heparin was chosen as the main anticoagulant for this study since it is the routine anticoagulant during CPB, allowing our experiments to be as close to the clinical situation as possible. To evaluate a possible influence of heparin on our results, heparinized blood samples were compared to blood anticoagulated with r-hirudin, an alternative anticoagulant for CPB. To our knowledge, no platelet activating effects have been reported for r-hirudin. In concordance with the literature (19), we found a distinct tendency for increased platelet P-selectin expression in heparinized blood. However, platelet aggregate and PMP formation as well as GP IIb/IIIa activation were not significantly influenced by heparin.

The use of hypothermia in cardiac surgery during CPB allows lower pump flow rates with less blood trauma and achieves better organ protection than normothermic CPB (1). Lowering the patient´s body temperature to at least mild hypothermia is applied in many patients. The threshold temperatures for increases of platelet activation markers P-selectin and activated GP IIb/IIIa in ADP-stimulated samples lie within and above the range of mild hypothermia. Therefore, an impact of hypothermia-induced platelet activation may not only occur during DHCA but in most patients undergoing cardiac surgery.

Application of a GP IIb/IIIa blocker like tirofiban has been reported to be safe and may have protective effects on platelets during CPB (20–22). This protective effect may be due to inhibition of the activation and interaction of platelets and leukocytes by GP IIb/IIIa blockade as it has been demonstrated during simulated ECC (23, 24). Hypothermia-induced platelet aggregates are probably linked by fibrinogen binding to GP IIb/IIIa (2, 5). As a result the application of a GP IIb/IIIa receptor blocker inhibits hypothermia-induced platelet aggregation and can decrease hypothermia-induced platelet damage (5). Therefore, GP IIb/IIIa blockade with a short-acting agent should be considered for platelet conservation against damage by ECC as well as for platelet protection against hypothermia. Recent advances may even allow to block GP IIb/IIIa in its activated state only (25).

Limitations of the study

As the described results were obtained from in-vitro experiments, conclusions for the in-vivo situation have to be drawn with caution. It would be desirable to evaluate platelets from patients undergoing cardiac surgery at reduced temperatures. However, in cardiac surgery hypothermia is always applied in combination with CPB, which itself induces activation and damage of platelets due to its artificial surfaces and shear stress. Therefore, if platelet activation and aggregation would be detected during hypothermic CPB, it would not be possible to clearly distinguish between hypothermia- or CPB-induced platelet activation and aggregation. A model such as the one used in this study is therefore essential to evaluate platelet function under defined in-vitro conditions, but can only suggest in-vivo relevance.

Conclusion

Mild hypothermia (31°C to 34°C) such as is often applied in cardiac surgery, leads to activation of platelets. In patients undergoing cardiac operations hypothermia-activated platelets and P-selectin bearing platelet microparticles may account for coagulation abnormalities, thromboemboli and inflammatory reactions. This study defines the critical temperatures, which lead to hypothermia-induced platelet activation and which indeed are relevant in cardiac surgery. To reduce the risk of platelet damage and loss through hypothermia while profiting from the positive effects of cooling in reducing organ ischemia, the application of short-acting GP IIb/IIIa blockers for platelet conservation during ECC should be considered.

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