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HYPOTHERMIA AND PLATELET DYSFUNCTION

BY

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NORMAL PLATELET PHYSIOLOGY

Platelets are essential for normal hemostasis. The main functions of platelets are adhesion to damaged blood vessel walls, aggregation to form a platelet plug, and promotion of fibrin clot formation. Platelet adhesion is primarily mediated by the adhesive molecule von Willebrand factor, which binds both to a specific platelet surface receptor (the glycoprotein [GP] Ib-IX complex) and to exposed subendothelial components.[1,2] Platelet-to-platelet aggregation is primarily mediated by fibrinogen binding to its platelet surface receptor (the GPIIb-IIIa complex).[3] Circulating platelets are normally in a resting state and, despite the presence of platelet surface GPIb-IX and GPIIb-IIIa complexes, they bind neither plasma von Willebrand factor nor plasma fibrinogen. *In vitro*, the cationic antibiotic ristocetin induces binding of von Willebrand factor to its receptor on GPIb,[1] but the *in vivo* analogue of ristocetin remains uncertain. Thrombin and other physiological platelet agonists (e.g. thromboxane A_2, adenosine diphosphate, and epinephrine) induce exposure of the fibrinogen receptor on the platelet surface GPIIb-IIIa complex.[3] Thromboxane A_2 is generated by platelets and is an important marker of platelet activation.[4] Platelet agonists such as thrombin and thromboxane A_2 also stimulate platelets to change shape, secrete the contents of their granules (e.g. ß-thromboglobulin, platelet factor 4, thrombospondin), and aggregate. Secreted thrombospondin binds to a receptor on the platelet surface membrane, as well as to fibrinogen, thereby stabilizing platelet-to-platelet aggregates.[5] GMP-140,[6] also referred to as P-selectin,[7] PADGEM protein,[8] and CD62,[9] is a component of the α granule membrane of resting platelets that is only expressed on the platelet plasma membrane after platelet activation and secretion. The platelet surface expression of

GMP-140 is therefore a very precise marker of platelet secretion. GMP-140 mediates adhesion of activated platelets to monocytes and neutrophils.[10-12] In contrast to its effect on GMP-140 and the GPIIb-IIIa complex, thrombin down-regulates the platelet surface expression of the GPIb-IX complex.[13-15]

HYPOTHERMIA AND PLATELET DYSFUNCTION

A hypothermia-induced hemorrhagic diathesis is known to occur in a number of clinical settings, including hypothermic cardiopulmonary bypass during cardiac surgery, other major surgery, multiple trauma, cold exposure, and neonatal cold injury.[16-22]

The hemorrhagic diathesis associated with hypothermic cardiopulmonary bypass during cardiac surgery is considered to be primarily a platelet function defect.[16,17,23] We have demonstrated that hypothermia results in both prolongation of the bleeding time and increased postoperative blood loss in patients undergoing cardiopulmonary bypass during cardiac surgery.[18,24] Consistent with this data, other investigators have recently reported that normothermic cardiopulmonary bypass during cardiac surgery results in less postoperative blood loss than hypothermic cardiopulmonary bypass.[25,26] In a clinical study we recently performed in 25 patients undergoing cardiopulmonary bypass with systemic hypothermia, one arm was kept warm with a water jacket throughout the intraoperative and postoperative periods while the temperature of the other arm reflected the systemic changes.[24] In the cold arm as compared to the warm arm, the bleeding time was significantly prolonged and the generation of thromboxane

B_2 (the stable metabolite of thromboxane A_2) was decreased in the shed blood emerging from the bleeding time wound.[24] This study[24] provided the first *in vivo* demonstration in humans that hypothermia results in a reversible decrease in platelet function.

However, cardiopulmonary bypass is a complex clinical setting in which to study the effects of hypothermia on platelet function, because even during normothermic cardiopulmonary bypass there is a platelet function defect.[17] We[27] and others[17] have demonstrated that hypothermia results in prolongation of the bleeding time in normal baboons. We have also reported that hypothermia prolongs the bleeding time in normal human volunteers.[28] In a recent study,[29] we verified *in vivo* that hypothermia inhibits platelet activation in normal human volunteers, as determined by five independent assays of the shed blood emerging from a standardized bleeding time wound: 1) up-regulation of platelet surface GMP-140 (reflecting α granule secretion) (Fig. 1A), 2) down-regulation of the platelet surface GPIb-IX complex (the von Willebrand factor receptor) (Fig. 1B), 3) platelet aggregate formation (Fig. 2), 4) generation of thromboxane B_2 (Fig. 3), and 5) the bleeding time (Fig. 4).

In a previous study in baboons,[27] we reported that the pathophysiological basis for hypothermia-induced platelet dysfunction was, at least in part, inhibition of the action of thromboxane synthetase, resulting in diminished thromboxane A_2 generation. We have recently confirmed and expanded upon these observations in humans.[29] Hypothermia resulted in lack of generation of thromboxane B_2 (the stable metabolite of thromboxane A_2) in humans both *in vitro* (Fig. 5B) and *in vivo* (Fig. 3).[29] Furthermore, in whole blood *in vitro*, hypothermia inhibits human platelet activation by thrombin and U46619 (a stable thromboxane A_2 analogue), as determined up-regulation of platelet surface GMP-140 (Fig. 6A), down-regulation of the platelet

surface GPIb-IX complex (Fig. 6B), and platelet aggregation (Fig. 5A).[29] Thus, in addition to their diminished generation of thromboxane A_2, hypothermic platelets are less reactive to thromboxane A_2 as well as to thrombin.

We have also demonstrated that rewarming hypothermic blood completely reverses the activation defect, as determined by four independent methods:[29] up-regulation of platelet surface GMP-140 (Fig. 6A), down-regulation of the platelet surface GPIb-IX complex (Fig 6B), platelet aggregation (Fig. 5A), and thromboxane B_2 generation (Fig. 5B). The reversibility of the hypothermia-induced inhibition of platelet activation suggests that rewarming a hypothermic bleeding patient may reduce the need for transfusions of platelets and other blood components, with their attendant risks and expense.[30] Furthermore, given that transfusion of platelets is relatively ineffective in the clinical setting of a hypothermia-induced hemorrhagic diathesis,[20] our data suggest that rewarming the patient will result in better function of transfused platelets. Every effort should be made to maintain normothermia in patients predisposed to hypothermia, such as during major surgery, multiple trauma, and the neonatal period.

In summary, hypothermia inhibits human platelet activation in whole blood both *in vitro* and *in vivo*. Rewarming hypothermic blood completely reverses the activation defect. These results suggest that maintaining normothermia or rewarming a hypothermic bleeding patient may reduce the need for transfusion of platelets and other blood components.

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FIGURE LEGENDS

Fig. 1. Hypothermia inhibits human platelet activation *in vivo*, as determined by up-regulation of platelet surface GMP-140 and down-regulation of the platelet surface GPIb-IX complex. After the skin temperature of the forearms of healthy volunteers was equilibrated to either 22°C, 32°C, or 37°C, a standardized bleeding time wound was performed. Local skin temperature was monitored by a surface thermometer placed within a few millimeters of the wound. The shed blood emerging from the bleeding time wound was fixed with 1% formaldehyde at 2 minute intervals until the bleeding stopped. The fixed samples were analyzed by whole blood flow cytometry for the platelet surface binding of monoclonal antibodies S12 (GMP-140-specific) (panel A) and 6D1 (GPIb-specific) (panel B). Maximum GMP-140 was defined as the binding of S12 to platelets in peripheral blood at 37°C after stimulation with thrombin 5 U/ml in the presence of 2.5 mM of the peptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP) (an inhibitor of fibrin polymerization and platelet aggregation). Maximum GPIb was defined as the binding of 6D1 to platelets in peripheral blood at 37°C. Each panel shows data from the same experiment on the same donor. At each time point for each temperature, the determination of platelet surface GMP-140 and GPIb was obtained from the same sample. The experiment is representative of 5 so performed. Reproduced with permission from Michelson et al. *Thrombosis and Haemostasis*, in press, 1993.

Fig. 2. Hypothermia inhibits human platelet activation *in vivo*, as determined by platelet aggregate formation. After the skin temperature of the forearms of healthy volunteers was

equilibrated to either 22°C, 28°C, or 32°C, a standardized bleeding time wound was performed. The shed blood emerging from the bleeding time wound was fixed at 2 minute intervals until the bleeding stopped. The number of platelet aggregates in the shed blood was determined by whole blood flow cytometry, as previously described.[29] The experiment is representative of 5 so performed. Reproduced with permission from Michelson et al. *Thrombosis and Haemostasis*, in press, 1993.

Fig. 3. Hypothermia inhibits human platelet activation *in vivo*, as determined by generation of thromboxane B_2 (the stable metabolite of thromboxane A_2). After the skin temperature of the forearms of healthy volunteers was equilibrated to either 22°C, 28°C, or 32°C, a standardized bleeding time wound was performed. Shed blood in 600 μL aliquots was collected continuously from the beginning to the end of the bleeding time. At each temperature, the first and last aliquots were assayed for thromboxane B_2. Data are mean $\pm$ S.E.M., n = 6 separate experiments. Reproduced with permission from Michelson et al. *Thrombosis and Haemostasis*, in press, 1993.

Fig. 4. Hypothermia inhibits human platelet activation *in vivo*, as determined by the bleeding time. The skin temperature of the forearms of healthy volunteers were equilibrated to 22°C, 28°C, 32°C, or 37°C. For the calculation of each data point the bleeding time was considered to be the mean of 2 duplicate standardized bleeding times. Data are mean $\pm$ S.E.M., n = 6. Asterisks indicate $p < 0.05$ by paired Student's t test, as compared to skin

temperature of 32°C (normothermia). Reproduced with permission from Michelson et al. *Thrombosis and Haemostasis*, in press, 1993.

Fig. 5. Reversibility of hypothermia-induced inhibition of platelet activation by thrombin, as determined by platelet aggregation (panel A) and thromboxane B_2 generation (panel B). Washed platelets (250,000/μL) in modified Tyrode's buffer, pH 7.4 were initially incubated at 37°C for 10 minutes, then the same platelets were cooled to 22°C over 30 minutes, and finally the same platelets were rewarmed to 37°C over 15 minutes. At each incubation temperature, an aliquot of platelets was activated with thrombin 0.16 U/mL. After 5 minutes, the digitized area under the aggregation curve (panel A) and the supernatant thromboxane B_2 concentration (panel B) were determined as previously described.[29] Data are mean $\pm$ S.E.M., $n = 4$ separate experiments. Asterisks indicate $p < 0.05$ by paired Student's t test, as compared to initial samples at 37°C. Reproduced with permission from Michelson et al. *Thrombosis and Haemostasis*, in press, 1993.

Fig. 6. Reversibility of hypothermia-induced inhibition of platelet activation in whole blood, as determined by thrombin-induced modulation of the platelet surface expression of GMP-140 (panel A) and GPIb (panel B). Blood was initially incubated at 37°C for 5 minutes, then the same blood was cooled to 22°C over 15 minutes, and finally the same blood was rewarmed to 37°C over 5 minutes. At each incubation temperature, an aliquot of whole blood was removed and activated at the 0 time point with thrombin 5 U/mL in the presence of GPRP 2.5 mM (both of which had been equilibrated to the temperature of the blood). Platelet activation

was stopped at various time points by the addition of 1% formaldehyde. The platelet surface binding of monoclonal antibodies S12 (GMP-140-specific) (panel A) and 6D1 (GPIb-specific) (panel B) was analyzed by whole blood flow cytometry. Maximum GMP-140 was defined as the binding of S12 after incubation with thrombin for 10 minutes at the first 37°C incubation. Maximum GPIb was defined as the binding of 6D1 at the first 37°C incubation in the absence of thrombin. Data are mean $\pm$ S.E.M., n = 3 separate experiments. Asterisks indicate $p < 0.05$ by paired Student's t test, as compared to samples at the initial 37°C incubation. Reproduced with permission from Michelson et al. *Thrombosis and Haemostasis*, in press, 1993.

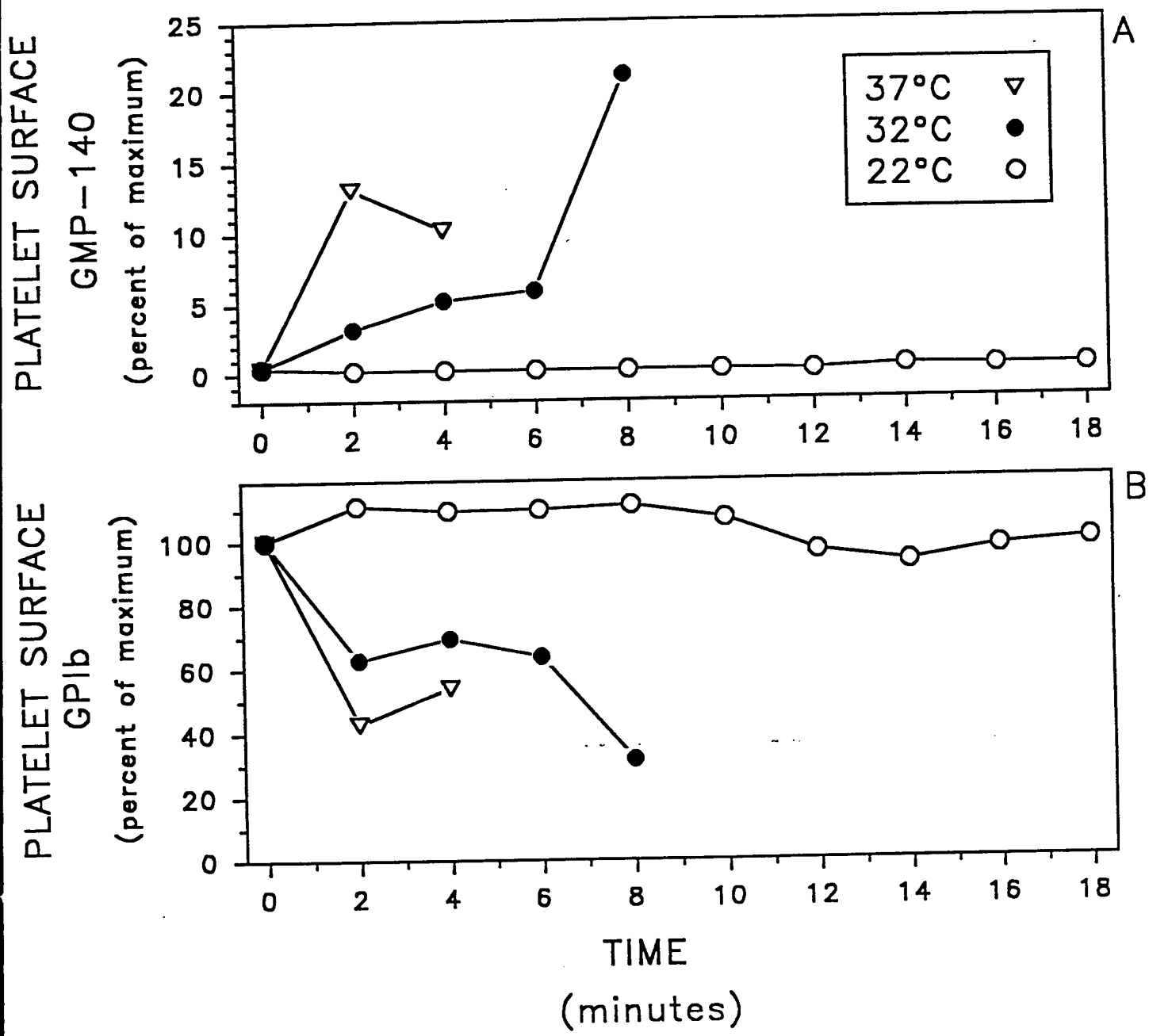
PLATELET SURFACE
GMP–140
(percent of maximum)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
A
37°C
32°C
22°C
PLATELET SURFACE
GPIb
(percent of maximum)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
B
TIME
(minutes)

Fig. 1

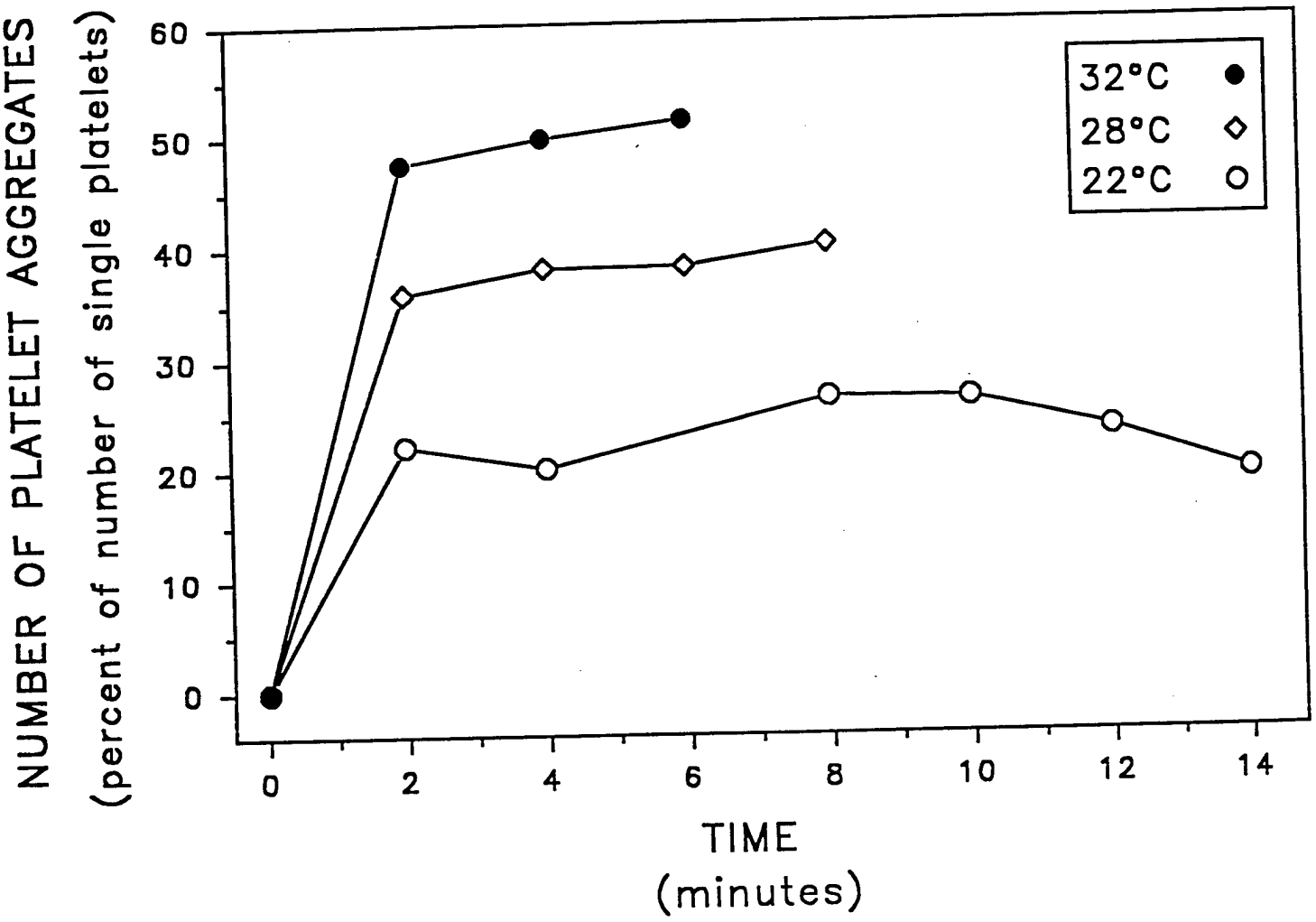
NUMBER OF PLATELET AGGREGATES
(percent of number of single platelets)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
TIME
(minutes)
32°C
28°C
22°C

Fig. 2

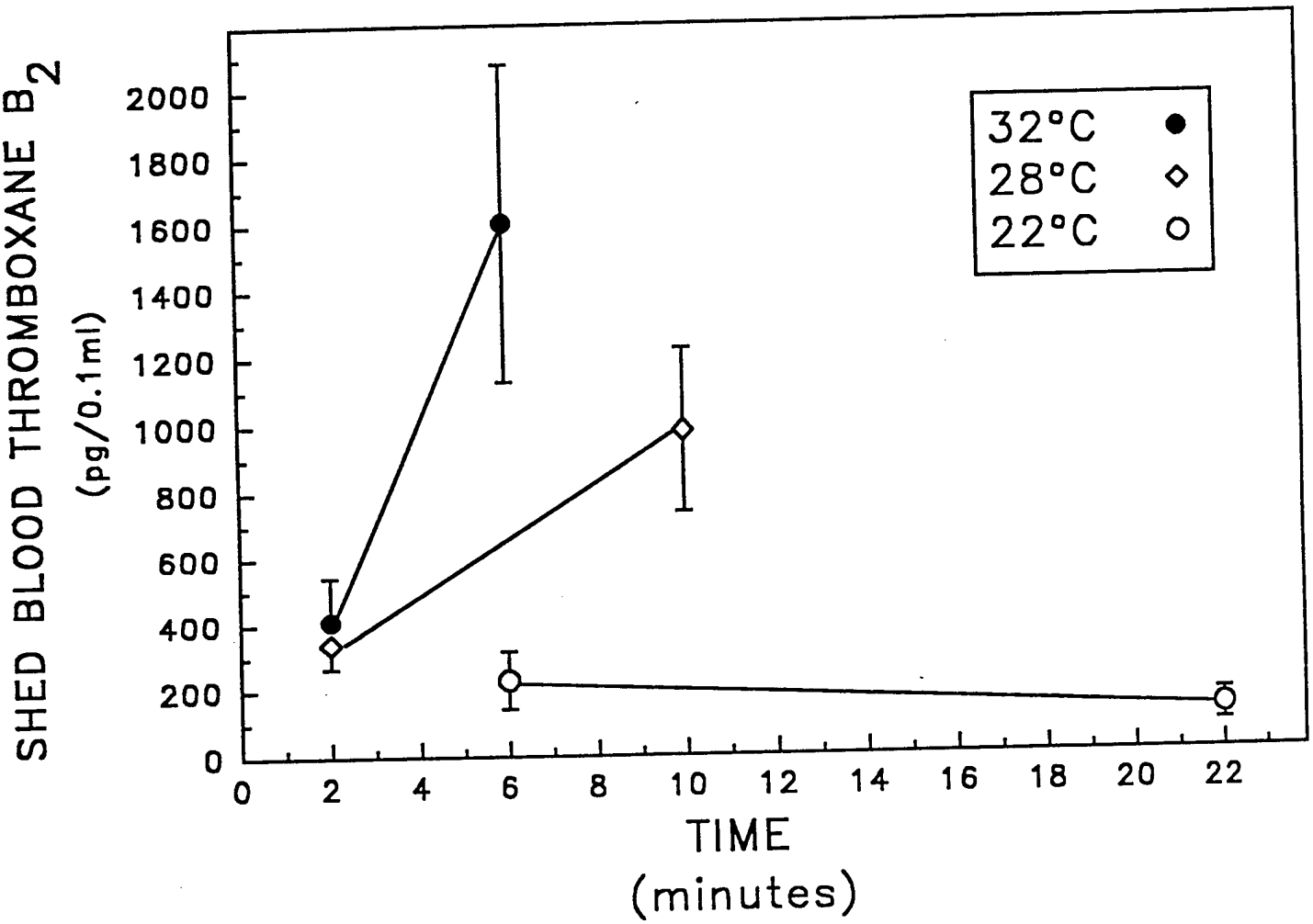
SHED BLOOD THROMBOXANE B_2
(pg/0.1ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
TIME
(minutes)
32°C
28°C
22°C

Fig. 3

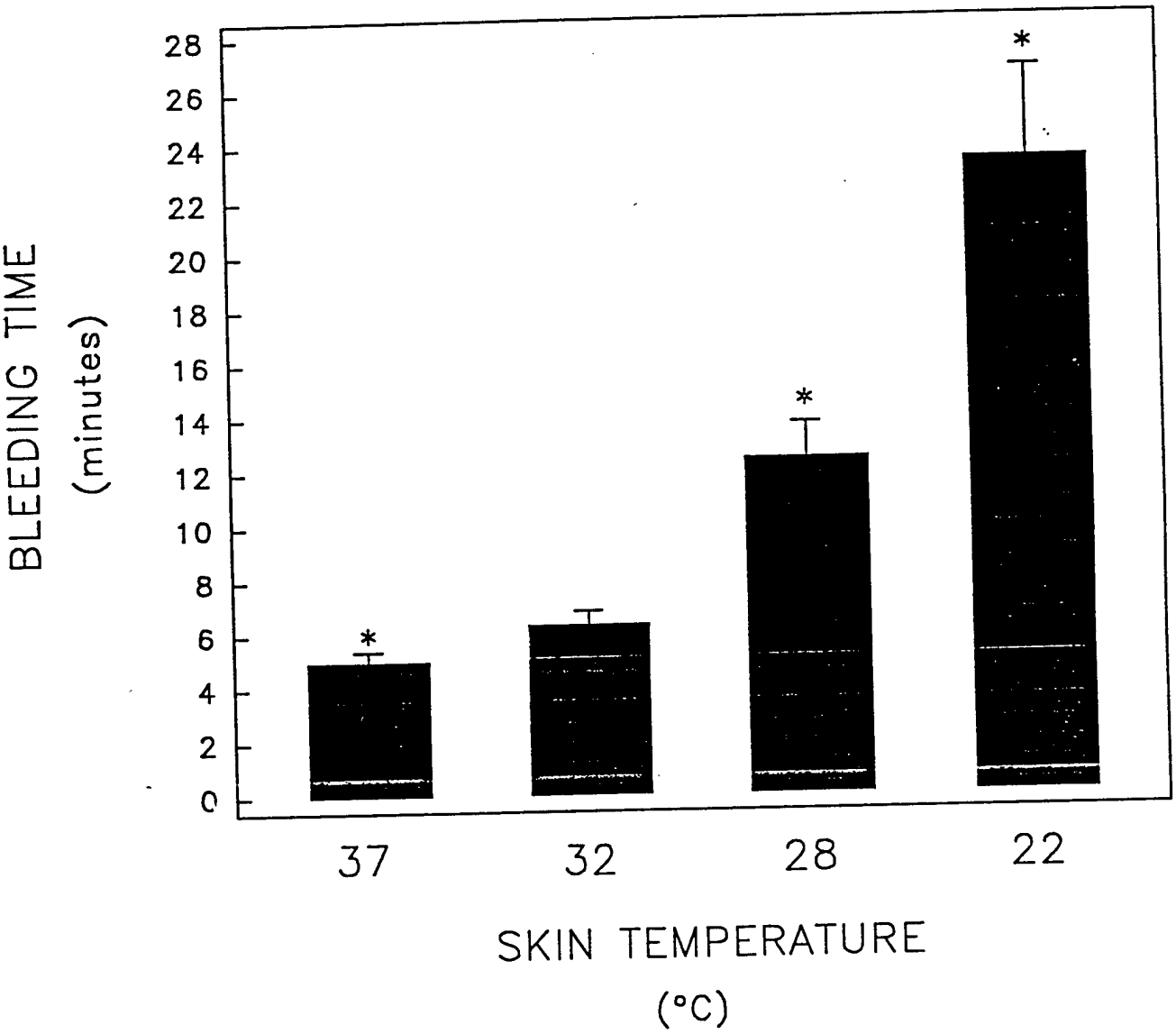
BLEEDING TIME
(minutes)
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
*
*
*
37
32
28
22
SKIN TEMPERATURE
(°C)

Fig. 4

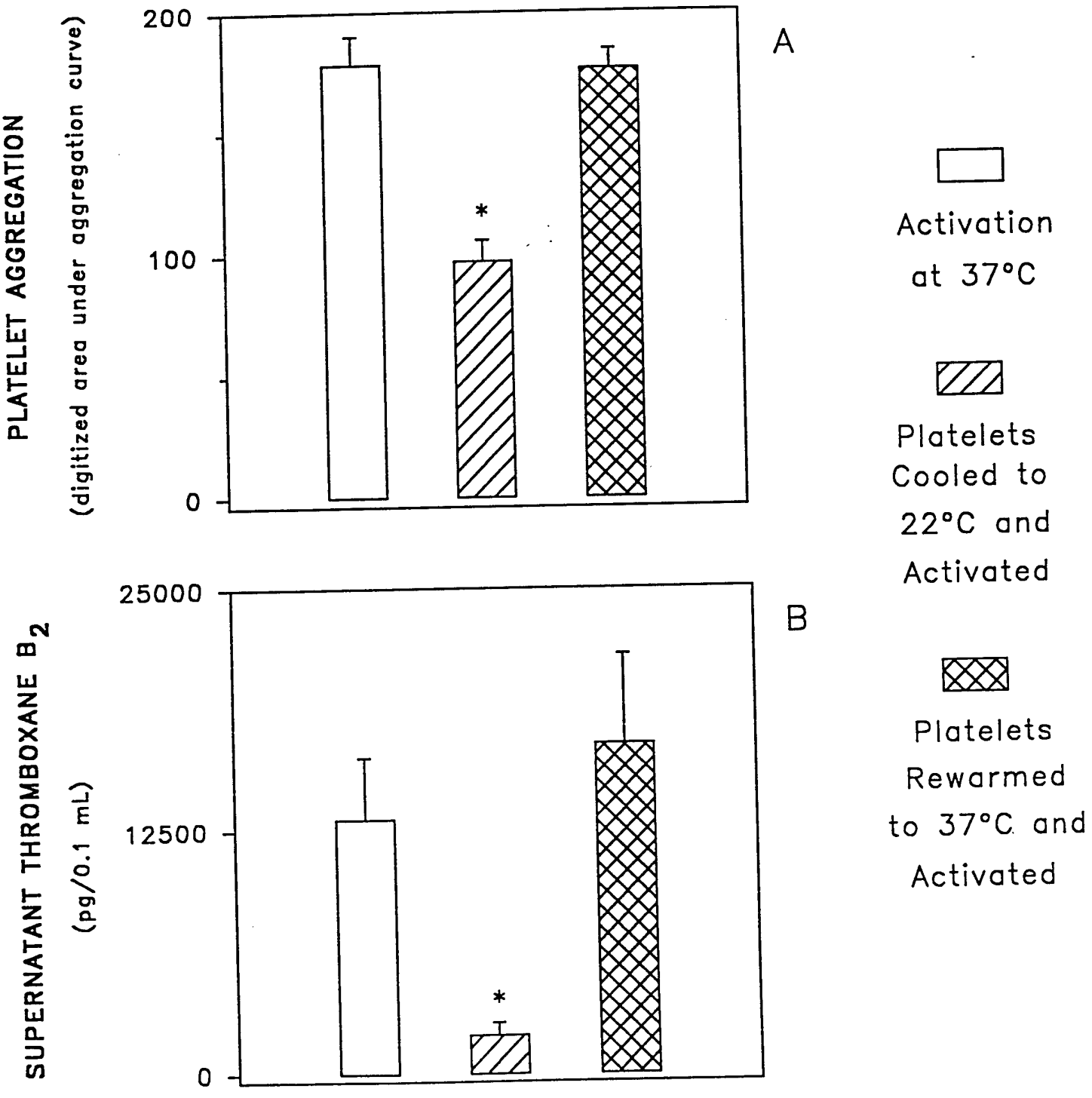
PLATELET AGGREGATION
(digitized area under aggregation curve)
200
100
0
*
A
SUPERNATANT THROMBOXANE B_2
(pg/0.1 mL)
25000
12500
0
*
B
Activation at 37°C
Platelets Cooled to 22°C and Activated
Platelets Rewarmed to 37°C and Activated

Fig. 5

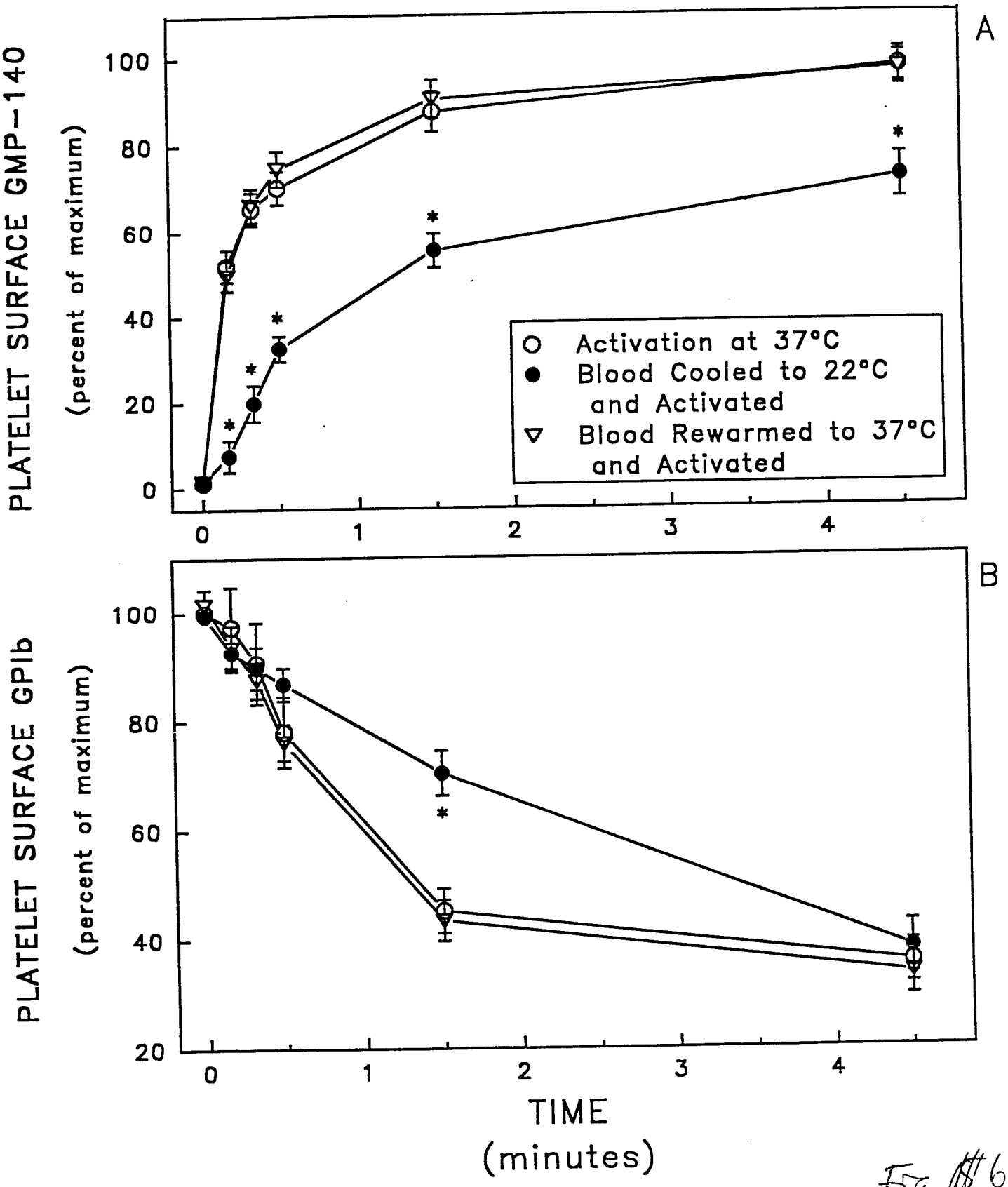
A
PLATELET SURFACE GMP-140
(percent of maximum)
100
80
60
40
20
0
0
1
2
3
4
Activation at 37°C
Blood Cooled to 22°C and Activated
Blood Rewarmed to 37°C and Activated
B
PLATELET SURFACE GPIb
(percent of maximum)
100
80
60
40
20
0
1
2
3
4
TIME
(minutes)

Fig. #6