0031-3998/89/2504-0405$02.00/0
PEDIATRIC RESEARCH

Vol. 25, No. 4, 1989
Printed in U.S.A.

Anticoagulant Effects of Heparin in Neonatal Plasma[1]

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ABSTRACT. Available data on the anticoagulant effects of heparin in neonatal plasma are scarce and conflicting: relative to adult plasma, neonatal plasma has been reported to show both resistance as well as sensitivity to heparin. We explored this apparent paradox by comparing how well heparin accelerated inhibition of exogenous thrombin and prevented thrombin generation in defibrinated neonatal and adult plasmas. Using amidolytic assays, we determined the effects of heparin on *1*) the neutralization of exogenous human α-thrombin and on *2*) the formation of endogenous thrombin activity after contact activation and recalcification. Neonatal plasma proved resistant to heparin (0.05 U/mL) during inhibition of added thrombin (15 NIH U/mL). Inhibition of thrombin in heparinized neonatal plasma became as efficient as in adult plasma only after raising the AT III activity to normal adult values. However, *de novo* generation of thrombin activity was very susceptible to inhibition by heparin, even in neonatal plasmas with physiologically low AT III levels. Peak thrombin activity generated in neonatal plasma in the absence of heparin was 50% or less of peak adult activity, and this already reduced ability of neonatal plasma to generate thrombin activity upon prothrombin activation was further decreased by heparin (0.05–0.2 U/mL). We conclude that due to the neonatal AT III deficiency, added thrombin is inactivated less effectively by heparin in neonatal than in normal adult plasma. Yet, the generation of thrombin activity is impaired in neonatal plasma and easily suppressed by heparin. We speculate that newborn infants may be resistant to heparin therapy during overt thrombotic disease, when neutralization of abnormal thrombin activity is the therapeutic goal. In contrast, lower plasma heparin levels may be required to prevent the formation of thrombin activity in newborn infants than in adult patients. (*Pediatr Res* 25:405–408, 1989)

Abbreviations

AT III, antithrombin III
PTT, partial thromboplastin time
PT, prothrombin time

Heparin is commonly used in sick newborn infants to prevent thrombus formation or treat thrombotic disease (1–5). However, available data on the action of heparin in neonatal plasma are scarce and conflicting. Relative to normal adult plasma, neonatal plasma has been reported to show both resistance as well as sensitivity to heparin (6). The present study was designed to explore this apparent paradox.

The coagulation system of the newborn infant is distinctly different from the adult system. In the healthy fullterm infant, the concentrations of prothrombin and other clotting factors as well as protease inhibitors, including AT III, are decreased to about 50% of adult values (7). The activities of these proteins are even lower in premature and sick infants (8, 9). AT III deficiency may cause resistance to heparin, as heparin acts primarily by catalyzing AT III-dependent reactions (10). By contrast, low concentrations of prothrombin and other clotting factors may result in sensitivity to heparin, as less heparin may be required to prevent thrombin generation. Heparin accelerates the neutralization of thrombin and inhibits its generation from prothrombin (11, 12). To investigate the reported "resistance" and "sensitivity" to heparin in the newborn, we compared the inhibition of exogenous thrombin and the generation of endogenous thrombin activity in heparinized neonatal and adult plasma as well as in neonatal plasma that had been supplemented with human AT III to achieve normal adult activities. This system was chosen because thrombin is one of the most important targets for the anticoagulant action of heparin (13).

MATERIALS AND METHODS

Materials. Arvin (Ancrod) was obtained from Connaught Laboratories, Toronto, Ont., Canada. Porcine intestinal mucosal heparin (Hepalean), 1000 USP U/mL, was purchased from Organon, Toronto, Ont., Canada, and the chromogenic substrate H-D-Phe-Pip-Arg-pNA (S-2238) from Kabi Vitrum, Stockholm, Sweden. Human α-thrombin was generously provided by John W. Fenton, New York State Department of Health, Albany, NY. Human AT III was a gift of Cutter Laboratories, Berkley, CA. Activated PTT reagent was purchased from Organon Teknika, Toronto, Ont., Canada. Fatty acid-free BSA and aprotinin (Trasylol) were products of Sigma Chemical Co., St. Louis, MO.

Collection and preparation of plasma samples. Cord plasma was used throughout this study. It can be considered to represent neonatal plasma on the 1st d of life (14). Cord blood was obtained during uneventful fullterm (37–41 wk of gestation) and premature deliveries (30–34 wk of gestation). A segment of the umbilical cord was double clamped immediately after birth, and blood was withdrawn from the umbilical vein into a polypropylene syringe containing 0.13 M sodium citrate and 100 U/mL of aprotinin solution (nine parts of blood, one part anticoagulant). Normal adult blood samples were obtained from 20 healthy donors (10 males, 10 females), and anticoagulated in the same fashion. Blood was also collected from 10 adult patients on warfarin medication.

Platelet-poor plasma was prepared by centrifugation at 3000 × *g* for 20 min at 4°C and stored at −70°C in polypropylene

Received June 21, 1988; accepted November 23, 1988.
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Supported in total by a grant from the Canadian Heart and Stroke Foundation. M. A. is a Scholar of the Canadian Heart and Stroke Foundation. B. S. was a Research Fellow of the Medical Research Council of Canada.
[1] Presented in part at the Annual Meeting of the Society for Pediatric Research, Anaheim, CA, 1987.

tubes until assayed. Plasmas of healthy and orally anticoagulated adults were pooled to obtain normal and abnormal adult plasmas, respectively. Plasmas of fullterm infants were either pooled (five cords per pool) or tested individually, as specified. Only cord plasmas with normal activated PTT (7, 8) were used in this study. The defibrinated plasma contained no greater than 20 μg/mL of fibrin-fibrinogen degradation products and did not inhibit a 2-U thrombin clotting time.

Amidolytic assay of thrombin inhibition. Thrombin was diluted in a buffer consisting of 0.05-M Tris, 0.15-M NaCl, pH 7.4, with 10 mg/mL BSA, to a final concentration of 15 NIH U/mL (150 nM). Defibrinated plasma (0.1 mL) was incubated with thrombin solution (0.1 mL) at 37°C. Both reagents were prewarmed. We determined that no measurable prothrombin consumption occurred during the incubation of plasma and thrombin. Aliquots (0.025 mL) of the thrombin plasma mixture were removed at timed intervals (up to 2 min) into 0.775 mL of a 0.16-mM S-2238 solution in 0.036-M sodium acetate, 0.036-M sodium barbiturate, 0.145-M sodium chloride, 0.1 mg/mL BSA, pH 7.40, which had been preincubated at 37°C. After a 10-min incubation at 37°C, the amidolysis of S-2238 was stopped by the addition of 0.2 mL acetic acid (50%); the absorbance was read at 405 nm. On some occasions, heparin (0.05 U–0.2 U/mL) and AT III (0.5 U/mL) were added to the test plasma before defibrination. The coefficient of variation for day-to-day measurements of total amidolytic activity after the addition of thrombin to normal adult plasma was less than 10%.

Amidolytic assay of thrombin generation. Total amidolytic thrombin activity after contact activation of plasma was quantitated by modifying a previously described test principle (15). Plasma was defibrinated by incubation with Arvin (0.18 U/mL of plasma) for 10 min at 37°C. Unless otherwise indicated, plasma will refer to defibrinated plasma. The initial fibrin clot was removed with a wooden applicator stick. After a further 5-min incubation at room temperature, any residual clot was similarly removed. The defibrinated plasma was kept at 4°C and used within 30 min. Activated PTT reagent (0.05 mL) was added to 0.1 ml of defibrinated plasma, and the mixture was incubated at 37°C for 3 min. Subsequently, 0.05 mL 40 mM $CaCl_2$ in 0.036-M sodium acetate, 0.036-M sodium barbiturate, 0.145-M sodium chloride, 0.1 mg/mL BSA, pH 7.40 was added.

After the addition of $CaCl_2$, 0.025-mL aliquots of the mixture were subsampled at timed intervals into tubes containing 0.475 ml 5-mM EDTA each, preincubated at 4°C. An aliquot (0.025 mL) of the EDTA-plasma mixture was transferred immediately into 0.775 mL of the above S-2238 solution. The amidolysis of the chromogenic substrate was measured as described for the assay of thrombin inhibition. Pretesting had confirmed that for up to 10 min the absorbance at 405 nm was directly proportional to the length of time that the thrombin-containing plasma EDTA sample was exposed to S-2238.

Total amidolytic thrombin activity was measured at least every 30 s for a total of 3 min, and every 15 s when peak amidolytic activity appeared. On some occasions, heparin (at varying concentrations from 0.05 to 0.4 U/mL) and AT III (0.5 U/mL) were added to the plasma before defibrination. The coefficient of variation for day-to-day measurements of peak amidolytic activity in adult pooled normal plasma was less than 5% in the absence of heparin and less than 10% if heparin was present in the plasma sample.

Amidolytic activity of the α_2-macroglobulin-thrombin complex. Thrombin bound to α_2-macroglobulin retains amidolytic activity, which contributes to the total amidolytic activity, as measured under the outlined assay conditions of thrombin inhibition and thrombin generation (11). To obtain a more precise estimate of free thrombin activity, we quantitated the amidolytic activity of the α_2-macroglobulin-thrombin complex as follows: At timed intervals, aliquots of the reaction mixture in both the thrombin inhibition and generation tests were subsampled into a solution containing heparin (20 U/mL plasma) and AT III (3.36 U/mL plasma) to rapidly and completely inactivate free thrombin in the sample. The residual amidolytic activity was then determined in the spectrophotometer as described for the total amidolytic activity above. The amidolytic activity of the α_2-macroglobulin-thrombin complex was subtracted from the total amidolytic activity, before comparisons were made between adult and neonatal thrombin inhibition.

Analysis. Amidolytic activities were expressed as percentage of peak normal adult values in keeping with the conventional way of reporting plasma activities of coagulation factors and protease inhibitors.

RESULTS

Effect of heparin on thrombin inhibition in neonatal and adult plasmas. Figure 1 illustrates the decay of the total amidolytic activity as well as the appearance of amidolytic activity associated with the α_2-macroglobulin complex in neonatal and adult pooled plasma after the addition of human α-thrombin. A large dose of thrombin (15 NIH U/L) was inactivated more slowly in neonatal than in normal adult plasma in the absence of heparin.

Figure 2 shows the relative resistance of neonatal plasma to the effects of heparin. No free thrombin activity was measurable in adult plasma containing 0.05 U of heparin/ml by 120 s, whereas thrombin inhibition in neonatal plasma was still incomplete (Fig. 2). To determine whether low neonatal AT III levels limited the anticoagulant action of heparin, we supplemented pooled fullterm plasma with 0.5 U/mL human AT III to achieve normal adult plasma AT III activities. This resulted in an improvement of neonatal thrombin inhibition by heparin (0.05 U/mL) such that inhibition of added thrombin in neonatal plasma became comparable to inhibition of added thrombin in normal adult plasma, containing the same amount of heparin (Table 1).

Effect of heparin on thrombin generation in neonatal and adult plasmas. After contact activation and recalcification, approximately 50% less thrombin activity was generated in neonatal than in normal adult plasma in the absence of heparin. The appearance of maximum thrombin activity was also delayed in neonatal plasma (Fig. 3). A similar reduction and delay of peak thrombin activity was observed in plasma from adult patients on warfarin (Table 2).

This already decreased ability of neonatal plasma to generate thrombin activity was further reduced by heparin (Fig. 3 and Table 2). At a low heparin concentration of 0.05 U/mL plasma,

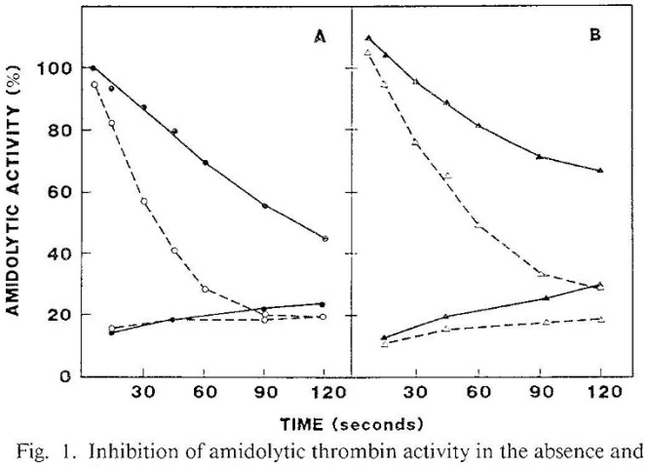

Fig. 1. Inhibition of amidolytic thrombin activity in the absence and presence of heparin in defibrinated and pooled normal adult (*A*) and fullterm cord plasma (*B*) after addition of 15 NIH U of human α-thrombin to 1 mL of plasma. The upper two curves in each panel represent total amidolytic activity; the lower two curves in each panel represent the amidolytic activity associated with the α-2-macroglobulin-thrombin complex. (See "Materials and Methods" for further details). *Solid lines*, no heparin; *broken lines*, 0.05 U heparin/mL plasma. Values are means of five measurements in each pooled plasma.

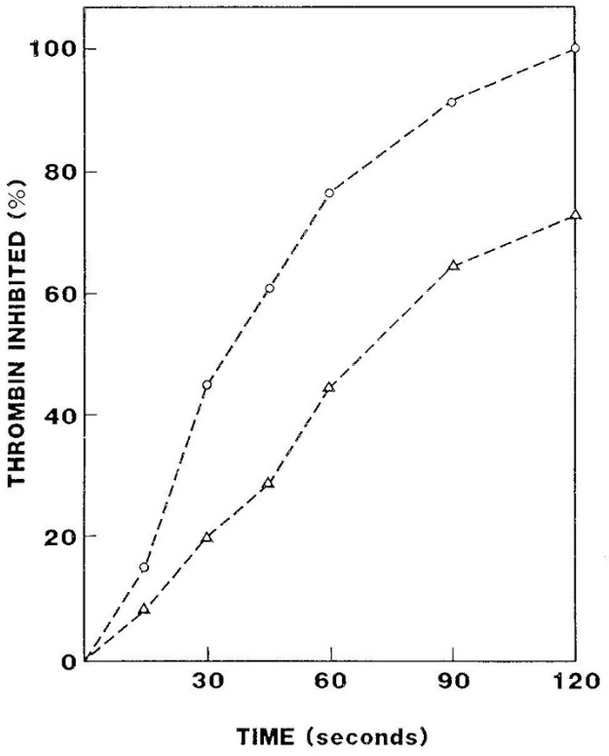

Fig. 2. Effects of heparin (0.05 U/mL) on thrombin inhibition in pooled normal adult (*open circles*) and fullterm cord plasma (*open triangles*); 100% thrombin activity refers to the activity detectable in the absence of heparin at the respective time interval after the addition of 15 NIH U of human α-thrombin to 1 mL of plasma. Values are means of five measurements in each pooled plasma.

Table 1. *Effect of added ATIII (0.5 U/mL) on thrombin inhibition by heparin (0.05 U/mL) in pooled neonatal plasma**

Incubation time (s)	% inhibition of thrombin activity†		
	Neonate	Neonate + ATIII	Normal adult
45	29	59	61
90	64	88	92

* Values are means of five measurements in each pooled plasma. The coefficient of variation for the amidolytic determination of thrombin activity was less than 10%.

† 100% thrombin activity refers to the activity detectable in the absence of heparin at the respective time interval after the addition of 15 NIH U of human α-thrombin to 1 mL of plasma.

which is typically achieved during heparin prophylaxis, peak thrombin activity in normal adult plasma was reduced by 15% and was not delayed, whereas peak thrombin activity in pooled fullterm plasma was reduced by 23% and delayed by 15 s. At a therapeutic concentration of 0.2 U/mL, heparin also inhibited and delayed the appearance of peak thrombin activity relatively more in neonatal than in normal adult plasma (Table 2). The addition of AT III to neonatal plasma further increased the effects of heparin (0.05 U/mL) on peak thrombin generation by 25%.

DISCUSSION

The low plasma concentrations of many procoagulants and protease inhibitors during the neonatal period have been extensively studied (7–9, 16). However, it is not fully understood how these low concentrations of coagulation proteins affect the action of heparin in neonatal plasma. Newborn infants have been reported to show resistance as well as sensitivity to heparin (6). We explored this apparent paradox by quantitating the inactivation of exogenous thrombin and the inhibition of thrombin generation in neonatal and adult plasmas. We chose to study the inhibition of thrombin rather than other coagulation enzymes, such as factor Xa, because of growing evidence that the inhibition of thrombin is critical to the antithrombotic effect of heparin (17).

In the present studies, 15 NIH U/mL of thrombin were inactivated more slowly in heparinized neonatal than in normal adult plasma. Inhibition of exogenous thrombin by heparin to the extent seen in normal adult plasma could only be achieved in neonatal plasma after supplementation with human AT III. This is consistent with our previous finding that the efficacy of heparin in neutralizing an intravenous bolus of thrombin was decreased in newborn piglets compared to mature pigs (18). This neonatal resistance to heparin could be overcome by supplementing the piglets with AT III (18). Like the human infant, newborn piglets are physiologically deficient in AT III.

In spite of the lower neonatal AT III levels, heparin was quite effective in inhibiting thrombin generation in neonatal plasma. The net result of adding prophylactic or therapeutic amounts of heparin to neonatal plasma was the appearance of very little

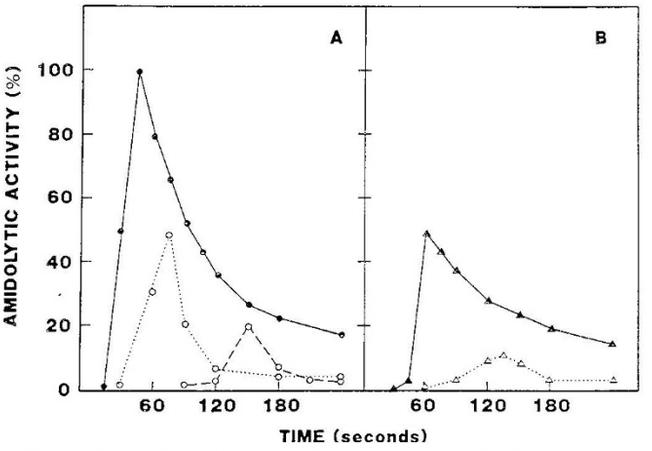

Fig. 3. Generation of amidolytic thrombin activity in the absence and presence of heparin in defibrinated and pooled normal adult (*A*) and fullterm cord plasma (*B*) after contact activation and recalcification. *Solid lines*, no heparin; *dotted lines*, 0.2 U heparin/mL plasma; *broken line*, 0.4 U heparin/mL plasma. Adult values are means of eight measurements in pooled plasma. Neonatal values are means of measurements performed in eight individual cord plasmas.

Table 2. *Effects of heparin on thrombin generation in pooled adult plasmas and individual neonatal plasmas*

Plasma from	Peak amidolytic thrombin activity (%): mean (range)		% reduction of peak thrombin activity by heparin
	No heparin	0.2 U/mL heparin	
Normal adults; eight measurements	100* (95–105)	49 (45–56)	51
Adults on warfarin; two measurements	34 (33–35)	5.5 (5–6)	84
Fullterm infants; n = 8	46 (34–64)	10 (4–16)	78
Preterm infants; n = 5	35 (26–49)	4.5 (0–5)	87

* 100% amidolytic activity corresponds to an absorbance reading of 0.865 at 405 nm.

thrombin activity compared to normal adult plasma. Our data suggest that during prothrombin activation, the decreased ability of neonatal plasma to generate thrombin activity outweighs the lack of AT III, as considerably less thrombin activity was generated in neonatal plasma in the absence of heparin. In fact, thrombin generation in unheparinized plasma from healthy full-term infants was comparable to thrombin generation in adult plasma which had been anticoagulated with 0.2 U/mL of heparin or which was obtained from patients on warfarin medication. This impaired ability of neonatal plasma to generate thrombin activity most likely reflects the lower plasma concentrations of vitamin K-dependent clotting factors, including prothrombin. Supportive evidence for this proposition comes from our observation that patients on warfarin, who have reduced functional activities of vitamin K-dependent clotting factors, also generated less thrombin activity with a greater lag time than healthy controls both in the absence and presence of heparin.

Neonatal plasma shows poor recovery of heparin activity in the antifactor Xa assay or the protamine neutralization test (19). This observation supports the concept of neonatal resistance to heparin. The notion of increased sensitivity has been based on the finding that heparin causes greater prolongation of the activated PTT and the PT in neonatal than in adult plasma (6). The present studies offer a plausible explanation for this apparent contradiction. Assay systems in which neonatal plasma appears resistant to heparin are critically dependent on the AT III concentration. They measure the inhibition of exogenous thrombin or factor Xa by the heparin-AT III complex. Unless neonatal plasma is sufficiently supplemented with AT III to correct fully the neonatal AT III deficiency, the anticoagulant action of heparin will be reduced in these assay systems (19). In contrast, assays such as the PTT, PT, or the thrombin generation test measure the effects of heparin on the formation of small amounts of endogenous thrombin activity. These assays appear to be affected more by the low levels of prothrombin and other procoagulant factors in neonatal plasma than by the low AT III levels.

Heparin is regularly used in many nurseries to prevent or treat neonatal thrombotic disease (1–5). Dosage recommendations for both prophylaxis and therapy are based entirely on anecdotal experience and not on controlled clinical trials of anticoagulant agents in the neonatal period (20). Our data suggest that newborn infants may be resistant to heparin therapy during overt thrombotic disease when the neutralization of abnormal thrombin activity is the therapeutic goal. By contrast, our data also suggest that considerably lower plasma heparin levels may be required to prevent *de novo* thrombin generation and possibly thrombosis in newborn infants than in older patients. These hypotheses will have to be tested in clinical trials aimed at finding the most beneficial and least harmful heparin doses to treat and prevent thrombotic disease in sick newborn infants.

Acknowledgments. We are grateful to Dr. J. Hirsh for a critical review of the manuscript. We thank Mrs. B. Lahie and Mrs. R. Phillis for their secretarial assistance.

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