PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 383–388 (1981)

Prostacyclin, Thromboplastin, and Antiheparin Activities of the Rat Aorta (41078)

CHUNG-HSIN TS'AO,[1] DIANE V. KRAJEWSKI, AND THERESA S. GALLUZZO

Department of Pathology, Northwestern University School of Medicine and Northwestern Memorial Hospital Chicago, Illinois 60611

Abstract. We detected three "hemostasis-relevant" activities in an isotonic Tris–saline buffer in which fragments of normal rat aorta had been incubated for 10 min. One activity inhibited adenosine diphosphate-induced platelet aggregation, the other promoted clotting of citrated plasma and caused aggregation of platelets in heparinized platelet-rich plasma, and the third suppressed the inhibitory effect of heparin on thrombin. These activities are represented by prostacyclin, thromboplastin, and an antiheparin of unknown nature. The platelet aggregation inhibiting activity in aorta-incubated buffer (AIB) disappeared upon storage or at high temperature, and was not present in AIB of aortas of aspirin-treated rats. Platelet aggregation of heparinized samples was inhibited by hirudin, citrate, high concentrations of heparin, and synthetic prostacyclin. The previously undescribed antiheparin activity appears to be distinct from both prostacyclin and thromboplastin.

Moncada *et al.* (1) demonstrated vessel wall release of prostacyclin (PGI_2) with a technique involving incubation of vessel segments in a buffer, and then testing the ability of the buffer to inhibit platelet aggregation induced by an aggregating agent. In analogous experiments using an isotonic Tris–saline buffer as the incubation fluid, we found two additional activities in buffer in which fragments of rat aorta had been incubated for 10 min. One is a thromboplastic activity, the other a heparin inhibitory activity.

Materials and Methods. Male Sprague–Dawley rats (Charles River, Wilmington, Mass., 250–350 g), anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal sodium, Abbott Lab., North Chicago; about 30 mg/kg), were bled by cardiac puncture. The blood was anticoagulated with either 1/10 vol of buffered sodium citrate (17.64 g trisodium citrate and 7.88 g citric acid per liter) or sodium heparin (mucosal heparin, Upjohn Co., Kalamazoo, Mich.; 2.5 U/ml blood). Citrated and heparinized platelet-rich and platelet-poor plasmas (cPRP, hPRP, and cPPP and hPPP) were prepared as previously described (2). Platelets in PRP were counted with a phase-contrast microscope. Washed platelet suspensions (WPS) were prepared according to Rossi (3). PRP and WPS were used in platelet aggregation experiments, citrated PPP was using in clotting tests.

While PRP and PPP were being prepared, laparotomy was performed and the abdominal aorta and inferior vena cava were severed to drain off remaining blood. The chest wall was then opened and the entire length of the descending thoracic aorta was removed. The lumen of the aorta was rinsed with isotonic saline and the surrounding connective tissue of the aorta was peeled off. The aorta was slit open and cut into 1 × 4-mm strips. Ten strips weighing 15–20 mg (wet wt) were placed in each milliliter of Tris (0.06 *M*)–saline (0.09 *M*) buffer (pH 7.5) at 24° for 10 min. The aortic fragments were removed and the buffer was tested either immediately, or after being left at room temperature for an hour or in a boiling waterbath for 15 sec to 30 min. Preliminary experiments showed absence of PGI_2 activity in all boiled samples of aorta-incubated buffer (AIB), but thromboplastic activity was highest in samples boiled for 3 or 5 min. Therefore, the 3-min boiled AIB was used in the experiments. In addition to aorta specimens prepared from normal rats, similar samples were prepared from two

[1] To whom all correspondence should be addressed.

0037-9727/81/030383-06$01.00/0

rats that had been given 80 mg of acetylsalicyclic acid (ASA, Sigma Co., St. Louis, Mo.) 2 hr prior to sacrifice. The ASA was suspended in water and administered via a gastric tube while the animal was anesthetized with ether.

Demonstration of PGI_2 activity. PGI_2 activity in the buffer was determined by a modification (4) of the method of Moncada *et al.* (1). Usually 5 and 0.5 μM of ADP (Sigma Co., dissolved in saline) were used to induce threshold platelet aggregation in citrated PRP (cPRP) and heparinized PRP (hPRP), respectively.

Demonstration of thromboplastic activity. Thromboplastic activity in the AIB was demonstrated by two methods. (A) Clotting of cPPP. cPPP, 0.1 ml, was mixed with 0.1 ml of plain Tris–saline buffer or AIB. After 3 min at 37°, 0.1 ml of calcium chloride (0.025 *M*, prewarmed to 37°) was added and the clotting time was determined by fibrometers (BioQuest Biological Lab., Cockeysville, Md.) in duplicate. (B) Aggregation of platelets in hPRP. 0.05 ml of buffer or AIB was delivered to 0.35 ml of hPRP in a cuvette. The mixture was stirred in a Chronolog platelet aggregometer for up to 6 min. In some experiments, 0.33 ml of hPRP was mixed with 0.02 ml of synthetic PGI_2 (kindly provided by Dr. John Pike of Upjohn Co., final conc. 0.1–0.5 ng/ml reaction mixture), ASA (f.c. 0.3 m*M*), heparin (f.c. 10 U/ml), buffered sodium citrate (0.1 *M*), or hirudin (Grade IV, Sigma Co., f.c. 10 and 20 U/ml) prior to the addition of AIB. In other experiments, 0.05 ml of bovine thrombin (Dade Corp., Miami; f.c. 0.25–12.5 U/ml) or a commercial tissue thromboplastin (Simplastin Automated, General Diagnostics, Morris Plains, NJ) was added to 0.35 ml of hPRP. The commercial thromboplastin was extracted from rabbit brain and lungs and contained $CaCl_2$. Each vial of the preparation was reconstituted as recommended by the manufacturer and further diluted 1:4 with saline.

Release of [^{14}C]serotonin (Amersham-Searle Co., Arlington Ht., Ill.) and formation of malondialdehyde (MDA) by hPRP exposed to AIB were determined with the method of Jerushalmy and Zucker (5), and Smith *et al.* (6), respectively. Serotonin release and MDA formation were also measured using collagen and arachidonic acid (AA, Nu-chek, Elysian, Minn.; f.c. 0.25 m*M*) as the stimuli.

Demonstration of antiheparin activity. Antiheparin activity of AIB was demonstrated by the use of a thrombin-sensitive synthetic substrate (H-D-phenylalanine–proline–arginine-5-amidoisophthalic acid, dimethyl ester, diacetate, or Phen–Pro–Arg-AIE; Dade Corp.). In the presence of heparin and antithrombin III (At-III), the amidolytic activity of thrombin will be inhibited. When the amidolytic activity of a known thrombin is established, the system can be employed to determine heparin (7) or At-III (8) activity by varying the concentration of these two components. We modified the method to assay antiheparin activity in AIB and in a commercial thromboplastin. Mucosal heparin (Upjohn) was diluted to 4.0 U/ml with saline and further diluted 10-fold with buffer, AIB, or a commercial thromboplastin. Since preliminary studies indicated that the antiheparin activity was better demonstrated in a stronger AIB than the one used in preceding experiments, we prepared AIB with 20 rather than 10 aortic fragments per milliliter. Five microliters of each heparin dilution was mixed with 200 μl human citrated plasma diluted 1:40 with a glycine buffer (Dade Corp., described in Ref. (7)) and warmed to 37°. Human plasma was the source of At-III. Then 50 μl of thrombin (1.0 U/ml) was added to the mixture. After a 1-min incubation, 2 ml of the synthetic substrate (0.14 mg/ml, prewarmed to 37°) was added. Uninhibited thrombin activity, expressed as percentage of original activity which was set at 100%, was determined with the "Protopath" (Dade Corp.) by kinetic measurement of release of the fluorophore AIE (excitation at 335 nm, emission at 430 nm). The presence of antiheparin in the test solution increases residual thrombin activity. AIB and commercial thromboplastin did not hydrolyze the synthetic substrate in the presence or absence of diluted human plasma, or inhibit At-III (8). No fibrin strands formed in a

mixture of thromboplastin and diluted human plasma after a 5-min incubation at 37°, nor did the mixture clot plasminogen-free bovine fibrinogen (97% clottable protein, Miles Lab., Elkhart, Ind.; 400 mg/dl; 1:1 mixture).

Results. Freshly prepared AIB was clear without macroscopically evident debris. It turned cloudy after being placed in a boiling waterbath for as little as 30 sec. The degree of cloudiness, judged by the naked eye, did not increase after 1-min boiling. Demonstration of PGI_2, thromboplastic, and antiheparin activities in AIB are described.

PGI_2 activity. AIB of normal rats completely inhibited ADP-induced platelet aggregation of cPRP and hPRP (Fig. 1). AIB left at room temperature for an hour, subjected to boiling, or obtained from aortas of ASA-treated rats had no inhibitory effect on platelet aggregation.

Thromboplastic activity.

(A) The recalcification time of rat cPPP, diluted 1:1 with Tris–saline buffer was 85.6 ± 13.3 sec (mean ± 1 SD; n = 12). When the buffer was replaced by AIB or heated AIB, the clotting time was shortened to 27.2 ± 2.9 sec and 24.5 ± 1.8 sec, respectively. The difference between the latter two values was not statistically significant.

(B) Freshly prepared AIB (50 μl) or AIB that had been left at room temperature for an hour did not cause any discernible change in PRP (350 μl) in the aggregometer. However, heated AIB samples from 28 normal and 2 ASA-treated rats induced aggregation of platelets in hPRP of normal and ASA-treated animals. The ASA was effective since platelets in hPRP of ASA-treated rats did not respond to a concentration of collagen that caused marked aggregation of platelets in hPRP of normal rats. None of the heated samples induced aggregation of platelets in cPRP or in an artificial medium. Representative hPRP responses to fresh and serially diluted heated AIB are shown in Fig. 2. Buffer alone, or buffer (0.5 ml) containing hPPP (0.02 ml), did not cause aggregation in hPRP after heating. The latter turned very cloudly after boiling.

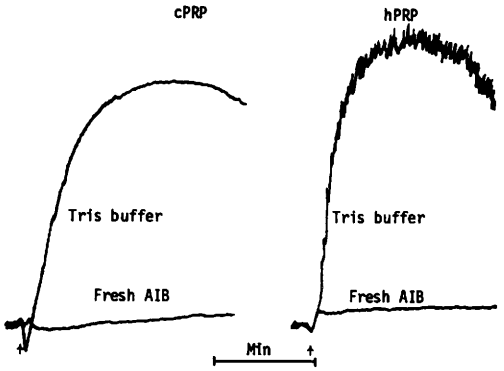

FIG. 1. Complete inhibition of ADP-induced aggregation of rat cPRP and hPRP by aorta-incubated buffer (AIB). AIB left at room temperature for an hour or placed in boiling waterbath for 3 min did not show this inhibitory activity. Neither did AIB of ASA-treated rats.

The lag period between the addition of heated AIB and aggregation of platelets in hPRP was long (2.4 ± 0.8 min). It was lengthened by synthetic PGI_2 or hirudin (Figs. 3A and B). ASA only slightly pro-

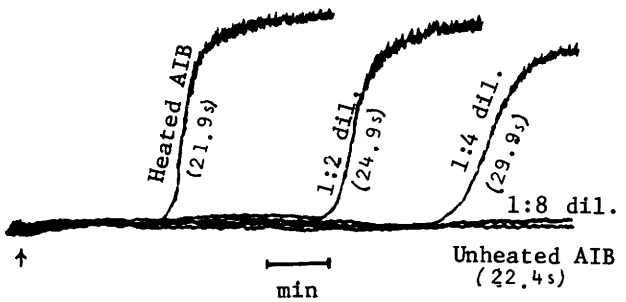

FIG. 2. Representative light transmission tracings of rat hPRP (350 μl) exposed to fresh and heated AIB (50 μl undiluted and 1:2, 1:4, and 1:8 diluted) in platelet aggregometer. Recalcification time of cPPP in the presence of these samples is stated in the parentheses.

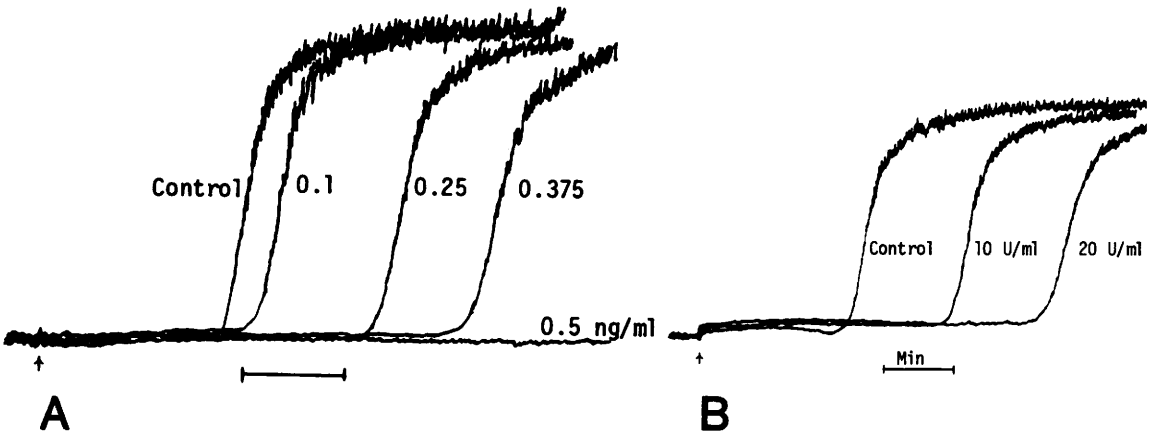

FIG. 3. Dose-dependent inhibition of AIB (heated)—induced aggregation of rat hPRP by synthetic PGI_2 (A) and hirudin (B).

longed the lag period (<30 sec); the same concentration of ASA completely suppressed arachidonic acid-induced aggregation. Heated AIB-induced aggregation was not observed in hPRP samples containing sodium citrate or additional heparin.

Aggregation of hPRP by heated AIB was accompanied by release of labeled serotonin, without significant formation of MDA (Table I). While release of serotonin induced by heated AIB was comparable to that induced by collagen, MDA formation stimulated by heated AIB was far less than by arachidonic acid or collagen.

Commercial thromboplastin also induced aggregation of rat hPRP; 3-min boiling had only a slight effect on its ability to aggregate hPRP or to clot cPPP (not shown). Commercial thrombin in a final concentration of 12.5 U/ml failed to induce aggregation of rat hPRP. Two percent of it, or 0.25 U/ml, caused marked aggregation of rat cPRP, followed by rapid clotting.

TABLE I. [^{14}C]SEROTONIN RELEASE AND MDA FORMATIONOF RAT hPRP EXPOSED TO HEATED AIB, AA, OR COLLAGEN

	Percentage [^{14}C]serotonin release	nM MDA/10^9 plts
Heated AIB	46 (3)[a]	0.82 (8)
AA	Not determined	4.33 (3)
Collagen	41 (3)	3.04 (3)

[a] The number of determinations is shown in parentheses.

Antiheparin activity. Heparin diluted in Tris–saline buffer exerted an 86% inhibition of the amidolytic activity of thrombin. Heparin diluted in heated AIB or heated thromboplastin caused comparable inhibition (79 and 86%, respectively). However, heparin diluted in unheated AIB or unheated thromboplastin caused significantly less inhibition (71%) than when diluted in Tris-saline buffer or heated samples. Consequently, residual thrombin activity was greater in the former than in the latter (Fig. 4). When heparin was not included in the assay system, thrombin caused equal hydrolysis of the substrate in samples containing unheated AIB or thromboplastin and in those containing Tris–saline buffer.

Discussion. We demonstrated three hemostasis-relevant activities in the Tris–saline buffer in which fragments of rat aorta were incubated for 10 min. One activity inhibited platelet aggregation, the other shortened clotting time of cPPP and promoted aggregation of platelets in hPRP, and the third inhibited the activity of heparin.

The finding of inhibiting platelet aggregation activity in AIB is consistent with observations of other investigators performing analogous experiments (1, 9–12). The activity is considered to be PGI_2. This notion is further supported by the findings of ab-

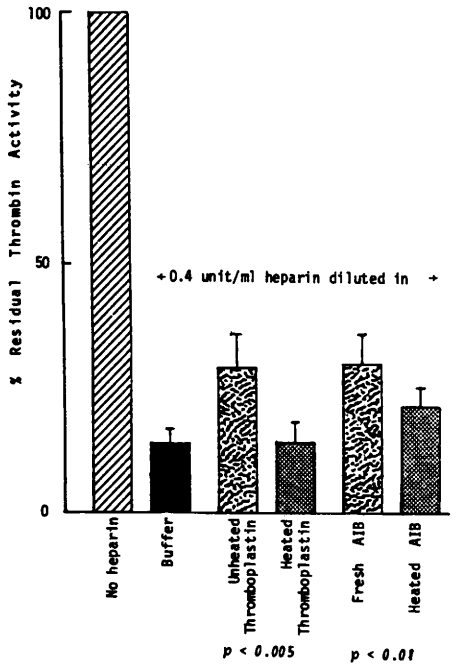

FIG. 4. Percentage residual thrombin activity determined in a system containing heparin and human plasma (to supply AT-III). Greater residual thrombin activity was found in samples whose heparin was diluted in fresh AIB (six determinations) or thromboplastin (five determinations) than in those whose heparin was diluted in buffer, or heated AIB, or heated thromboplastin. Difference in residual thrombin activity between unheated and heated samples is significant.

sence of inhibiting activity in media prepared with aortas of ASA-treated rats, and by the disappearance of the activity after heating. Inhibition of production by ASA and lability at high temperatures are well-known properties of PGI_2.

The aortic wall is known to contain thromboplastin (13–15). With peroxidase-conjugated antibodies raised against bovine lung thromboplastin, Zeldis *et al.* (16) localized thromboplastin to the intima of bovine aorta. Earlier, thromboplastic activity was demonstrated in extracts of homogenized vessels. Recently, the activity has been shown to be released from rat aortic tissue to autologous PRP and PPP; the activity appears to be affected by the fatty acid composition in the diet (17). Here, we showed the presence of thromboplastic activity in a buffer which had been incubated with fragments of aortas of normal and ASA-treated rats.

We used two methods to demonstrate thromboplastic activity: promotion of plasma clotting and induction of platelet aggregation. The former is an established technique to detect and quantitate thromboplastic activity (13–15). The latter was conceived on grounds that a given concentration of heparin, sufficient to prevent blood from clotting, would not inhibit all thrombin generated from plasma by the action of thromboplastin. The residual thrombin would induce platelet aggregation. Since a lower concentration of thrombin is required to induce platelet aggregation than fibrinogen–fibrin conversion, platelet aggregation induced by thromboplastin can be separated from plasma clotting. In addition, since divalent cations in the blood are not chelated in heparinized samples, there is no need for exogenous $CaCl_2$. The advantages of using platelet aggregation to study thromboplastic activity are twofold: (a) it represents another method with which thromboplastic activity can be evaluated, and (b) it permits investigation of effects of platelet-affecting substances (e.g., PGI_2, ASA) on the overall impact of thromboplastin on the hemostatic system.

When freshly prepared AIB failed to induce aggregation of platelets in hPRP, we suspected, among other possibilities, that the presence of PGI_2 in AIB had inhibited platelet aggregation. We chose a quick way (boiling) to inactivate PGI_2. Every heated AIB sample caused platelet aggregation. However, as indicated by additional work, the ability of heated AIB to induce platelet aggregation is not due merely to the destruction of PGI_2. Unheated AIB, left at room temperature for an hour and showing no PGI_2 activity, did not induce platelet aggregation. Furthermore, we excluded the possibility that the aggregation was induced by denatured protein particles in heated AIB, because heat-denatured plasma proteins in Tris–saline buffer did not cause platelet aggregation. It is not clear why heating of AIB would enable it to induce aggregation. There is little doubt that the aggregation of hPRP by heated AIB was a

result of generation of thrombin from plasma by thromboplastin released from rat aorta, because it was abolished or inhibited by sodium citrate, additional heparin, or hirudin, a specific inhibitor of thrombin. Had stronger hirudin been used (the amount of hirudin in the container did not allow us to make it stronger without altering the volume ratio of PRP, hirudin, and AIB), the aggregation might be completely inhibited.

It is not surprising the AIB-induced aggregation was accompanied by release of labeled serotonin, and was inhibited by PGI_2. It is unexpected that the aggregation was hardly affected by ASA. Both the absence of effect by ASA and the low levels of MDA formation appear to indicate that the arachidonic acid–prostaglandin pathway is only minimally involved in these AIB-induced rat platelet aggregation reactions. When PGI_2 production is inhibited while thromboplastic activity is not, the thrombogenic potentials of the vessel wall would be increased. This could explain the thrombogenecity of high doses of aspirin (18).

Gomperts and Zucker (19) found a heparin inhibitory activity in brain thromboplastin which parallels the procoagulant activity. Using a different technique, we also detected an antiheparin activity in a commercial thromboplastin as well as in AIB samples. Contrary to findings of Gomperts and Zucker, who noted the disappearance of both antiheparin and procoagulant activities in brain thromboplastin after incubation of 56° or higher temperatures, the procoagulant activity in our samples (AIB and thromboplastin) survived our heating treatment (3 min in a boiling water bath) whereas the antiheparin activity did not. The antiheparin activity appears to be distinct from the procoagulant activity. At the present time, neither the nature nor the function of vascular antiheparin is known. It may very well be a positively charged basic protein. Since the arterial wall does contain heparin-like substances (e.g., proteoglycans (20–22)), it is tempting to speculate that the antiheparin may play a role in the maintenance of the homeostasis of the arterial wall and in the overall function of the wall in hemostasis and thrombosis.

1. Moncada, S., Higgs, E. A., and Vane, J. R., Lancet **1**, 18 (1977).
2. Ts'ao, C., Amer. J. Pathol. **85**, 581 (1976).
3. Rossi, E. C., J. Lab. Clin. Med. **79**, 240 (1972).
4. Ts'ao, C., Holly, C. M., Serieno, M. A., and Galluzzo, T. S., Thrombos, Hemostas. **42**, 873 (1979).
5. Jerushalmy, A., and Zucker, M. B., Thrombos. Diathes. Haemorrh. **15**, 413 (1966).
6. Smith, J. B., Ingerman, C. M., and Silver, M. J., J. Lab. Clin. Med. **83**, 167 (1976).
7. Mitchell, G. A., Gargiulo, R. J., Huseby, R. M., Lawson, D. E., Pochron, S. P., and Schuanes, J. A., Thrombosis Res. **13**, 47 (1978).
8. Mitchell, G. A., Hudson, P. M., Huseby, R. M., Pochron, S. P., and Gargiulo, R. J., Thrombosis Res. **12**, 219 (1978).
9. Villa, S., and de Gaetano, G., Prostaglandins **14**, 1117 (1977).
10. Remuzzi, G., Cavenaghi, A. E., Meccas, G., Donati, M. B., and de Gaetano, S., Lancet **2**, 1195 (1977).
11. Hornstra, G., Haddeman, E., and Don, J. A., Thrombosis Res. **12**, 367 (1978).
12. Buchanan, M. R., Dejana, E., Cazenva, J. P., Mustard, J. F., and Hirsh, G., Thrombosis Res. **16**, 551 (1979).
13. Astrup, T., Albrechtsen, O. K., Classen, M., and Rusmussen, J., Circ. Res. **7**, 969 (1959).
14. Astrup, T., and Buluk, K., Circ. Res. **13**, 253 (1963).
15. Kirk, J. E., Proc. Soc. Exp. Biol. Med. **109**, 890 (1960).
16. Zeldis, S. M., Nemerson, Y., Pitlik, F. A., and Lentz, T. L., Science **175**, 766 (1972).
17. Hornstra, G., and Hemker, H. C., Haemostasis **8**, 211 (1979).
18. Kelton, J. G., Hirsh, J., Carter, C. J., and Buchanan, M. R., J. Clin. Invest. **62**, 892 (1978).
19. Gomperts, E. D., and Zucker, M. R., Thrombosis Res. **12**, 105 (1977).
20. Murata, K., Nakazawa, K., and Hami, A., Atherosclerosis **21**, 93 (1975).
21. Ts'ao, C., Eisenstein, R., and Schumacher, B., Proc. Soc. Exp. Biol. Med. **156**, 162 (1977).
22. Vijayagopal, P., Radhakrishnamurthy, B., Srinivasan, S. R., and Berenson, G. S., Lab. Invest. **42**, 190 (1980).

Received June 27, 1980. P.S.E.B.M. 1981, Vol. 166.