dog kidney may represent the physiological prejunctional counterpart to the specific DA receptors of the canine renal vasculature (*1*). These receptors were identified as the result of pharmacological responses to exogenous DA agonists and antagonists. The location of these DA-containing elements at the glomerular vascular poles throughout the cortex places them contiguous not only with vessels that can regulate blood flow through the glomeruli, but also with the juxtaglomerular apparatus. If DA is released from these stores as the result of physiological stimuli, a dopaminergic component in the normal regulation of renal blood flow and renin release may exist.

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8. The kidneys of three dogs anesthetized with pentobarbital (30 mg/kg) were exposed by flank incision and retroperitoneal dissection. Immediately prior to their removal, the kidneys were perfused with 0.9 percent saline at 4°C. Immediately after removal, the kidney was placed in a tray set on ice. Cross sections (1.5 mm thick) of dog kidney were cut, then divided into quarters. They were rapidly frozen in liquid nitrogen–solid slush [L. J. Roth, I. M. Diab, M. Watanabe, R. J. Dinerstein, *Mol. Pharmacol.* **10**, 986 (1974)] and then stored in liquid nitrogen. For histofluorescence measurements, kidney cross sections were warmed to −30°C in a cryostat and sectioned at 10 μm. The sections were freeze-dried for 5 days at −65°C in a chamber that contained P_2O_5 and was evacuated with a mechanical oil pump. The sections were dry-mounted on Formvar-coated slides and gassed for 4 hours at room temperature [R. Hakanson and F. Sundler, *J. Histochem. Cytochem.* **22**, 887 (1974)] with formaldehyde that had been equilibrated with air at 60 percent relative humidity.
9. Microspectrofluorometry was performed with epi-illumination. Monochromatic light was focused through quartz optics and a reflecting objective (Beck) onto embedded tissue sections. Fluorescence emission was collected by way of the same objective and focused through a Leitz MPV photometer tube in which a limiting diaphragm could be set to frame the tissue region or cellular structure to be measured. Double-grating monochromators (Schoeffel) were used on both the excitation and emission arms of the microfluorometer to reduce stray light from the solid tissue sample. Photon counting was used to improve sensitivity. Spectra obtained were corrected with calibration curves. [C. A. Parker and W. T. Rees, *Analyst* **85**, 587 (1960)]. The catecholamine-specific fluorescence was identified by measuring the fluorescence spectrum from an individual neuronal element. Contributions to this spectrum from nonspecific tissue fluorescence and light scatter were removed by subtracting an identical scan of a nearby nonfluorescing glomerular capillary.
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Heparin Neutralization of PGI_2: Effects upon Platelets

Abstract. *Heparin neutralizes the inhibitory effect of prostacyclin (PGI_2) on platelet aggregation. The PGI_2-induced enhancement of platelet cyclic adenosine monophosphate levels is also inhibited. The mechanism appears to involve a direct interaction in which heparin neutralizes the inhibitory effects of PGI_2 on platelet aggregation but, at the same time, does not lose its own anticoagulant activity. These findings may explain instances in which heparin infusions have been reported to produce hyperaggregation of platelets, thrombotic episodes, and thrombocytopenia in patients.*

Direct roles for intact endothelium in maintaining the fluidity of blood are becoming increasingly apparent (*1*, *2*). Endothelium of several species has been found to synthesize and release prostacyclin (PGI_2), a substance that possesses a marked inhibitory activity for platelet aggregation (*3*–*7*). Recently, it has been discovered that prostacyclin is a circulating hormone that is generated continuously in the lungs (*8*). Release of PGI_2 from endothelium may prove to be a highly significant physiologic event in inhibiting platelet-platelet and platelet-endothelial interactions, and thus it may play a central role in the prevention of venous and arterial thrombosis. It has been reported that an increase in platelet cyclic adenosine monophosphate (AMP) may be involved in PGI_2-induced inhibition of platelet aggregation (*9*).

Heparin is considered a clinically useful antithrombotic agent. Over the years, however, there has been controversy with respect to the effects of heparin on platelet function. Although heparin has been reported to cause inhibition of platelet aggregation (*10*, *11*) and to inhibit the epinephrine-induced release reaction of platelets treated with adenosine diphosphate (ADP) (*10*–*12*), there have been reports of heparin causing hyperaggregation of platelets (*13*, *14*), paradoxical thrombosis (*15*), and thrombocytopenia (*16*). Mechanisms of hyperaggregation and thrombocytopenia induced by heparin have remained controversial. A direct activating effect of heparin on platelets (*13*, *17*), molecular heterogeneity, and variation of commercial sources (*14*, *18*) has been considered responsible for this phenomenon. Furthermore, development of a heparin-induced immune mechanism causing thrombocytopenia has been proposed (*19*). These controversies are appropriately depicted in the review of Wessler and Gitel (*17*) as they state "it is still not clear whether heparin in vivo prevents or facilitates platelet aggregation." We undertook a study of heparin on PGI_2-induced inhibition of platelet aggregation. The results indicate that heparin has the capacity to neutralize effectively the inhibitory activity of PGI_2 on platelet aggregation as well as on the enhancement of platelet synthesis of cyclic AMP that ordinarily parallels inhibition of platelet aggregation.

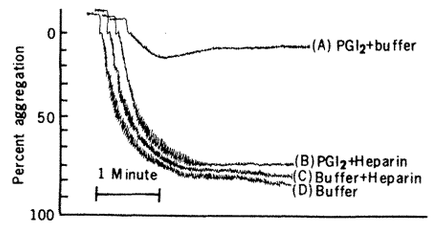

Fig. 1. Neutralization of PGI_2 by prior incubation with heparin. A 1:1 mixture of PGI_2 (0.02 μ*M*) and heparin (20 unit/ml) was incubated for 10 minutes at 37°C. For controls, buffer was incubated alone or with PGI_2 or heparin. A 0.2-ml sample of this mixture was added to 0.2 ml of PRP in a cuvette in the aggregometer. A 0.1-ml sample of ADP (10^{-5}*M* was then added and the aggregation was recorded. (Results represent the findings of five experiments.)

Synthetic PGI_2 was a gift from Dr. John E. Pike of the Upjohn laboratories. It was obtained as the sodium salt and dissolved in modified (Ca^{2+},Mg^{2+}-free) Tyrode solution (buffer). All experiments were performed with platelet-rich plasma (PRP) prepared from the blood of

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normal human volunteers who did not ingest pharmaceuticals during the previous week; one part of 3.8 percent sodium citrate was added to each nine parts of blood. The *p*H of the PRP was 7.4. Thrombin (1000 unit/ml, Bovine Topical) was obtained from Upjohn. Sodium heparin (1000 unit/ml) from various sources (*20*) was used. All of them demonstrated similar PGI_2 neutralizing activity. These preparations of heparin have undergone various clinical trials in human subjects and are now being used for treatment of patients with thromboembolic disorders. Adenosine diphosphate was obtained commercially (Sigma, grade III, sodium salt). Platelet aggregation studies were performed turbidometrically (*21*) with a Biodata platelet aggregometer. Platelet cyclic AMP was assayed by a commercially available protein binding assay (Amersham/Searle) by the method of Tateson *et al.* (*9*).

Prostacyclin and heparin were tested separately and in combination for their effects on platelet aggregation induced by added ADP. The PGI_2 alone markedly inhibited platelet aggregation by ADP (Fig. 1, curve A). Heparin alone produced no significant effect on ADP-induced platelet aggregation compared to a buffer control (curve C). However, a mixture of PGI_2 and heparin incubated for 10 minutes showed neutralization of PGI_2 inhibition of platelet aggregation (curve B). The same phenomenon of blockage of the inhibitory effects of PGI_2 was observed when collagen or ristocetin (not illustrated) were substituted for ADP as inducers of platelet aggregation.

Although Fig. 1 represents the studies with 20 units of heparin per milliliter, in other studies (not illustrated) lower concentrations of heparin, including 10, 5, 2.5, 1.25, and 0.5 unit/ml, were used. The blocking of PGI_2 effect by heparin was seen at concentrations as low as 2.5 unit/ml; concentrations lower than this showed only minimal or no blocking effects.

Table 1. Neutralization of PGI_2 by heparin in platelet-rich plasma (PRP). To 0.2 ml of PRP, 0.1 ml (10 unit/ml) of heparin and then 0.1 ml (0.004 μM) of PGI_2 were added. For controls, buffer replaced PGI_2 or heparin, or both. The mixtures were then incubated for 10 minutes. A 0.1-ml sample ($10^{-5} M$) of ADP then was added to each mixture and the degree of platelet aggregation was determined. The percentage of aggregation is the percentage of transmission (arbitrary units) recorded 1 minute after addition of ADP.

Incubation mixture				Aggregation (%)
Buffer	PGI_2	Heparin	ADP	
+	−	−	+	78
+	−	+	+	90
+	+	−	+	23
−	+	+	+	52

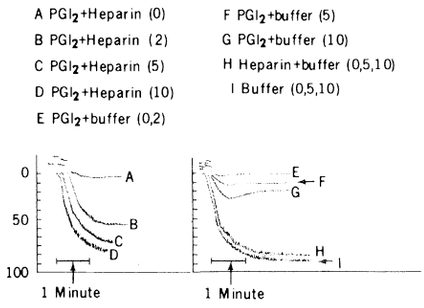

Fig. 2. Time-dependent reaction between PGI_2 and heparin. A mixture of PGI_2 (0.02 μM) and heparin (20 unit/ml) (1:1) was incubated. At 0, 2, 5, and 10 minutes (indicated by numbers in parentheses), 0.2 ml of the incubation mixture was added to 0.2 ml of PRP placed in the cuvette of the aggregometer. A 0.1-ml sample of ADP ($10^{-5} M$) was then added, and the aggregation pattern was recorded. (The findings represent the results of five experiments.)

In another experiment, equal volumes of PGI_2 and heparin solutions in buffer were combined and incubated. After different intervals of 0, 2, 5, and 10 minutes, a sample from an incubation mixture was added to PRP, followed by the immediate addition of ADP to initiate aggregation of platelets. Heparin at 0-minute incubation with PGI_2 failed to block the PGI_2 inhibitory effect (Fig. 2, curve A). After 2 minutes of incubation of heparin with PGI_2, there was some diminution of the PGI_2 effect (curve B), and, as the time of incubation of heparin with PGI_2 was increased to 10 minutes, heparin neutralized completely the ability of PGI_2 to inhibit platelet aggregation (curve D). This experiment also suggests that heparin neutralizes PGI_2 directly rather than by acting as a competitive inhibitor.

In other experiments, PGI_2 and heparin were added directly to PRP simultaneously, and the mixture was incubated for 10 minutes. Then ADP was added in order to determine whether neutralization of PGI_2 by heparin occurred in this system (Table 1). In the presence of buffer replacing PGI_2 and heparin, ADP produced 78 percent aggregation. Heparin with ADP produced 90 percent aggregation, an enhancing phenomenon reported by several other investigators (*13, 14*). The PGI_2 produced only 23 percent aggregation in the absence of heparin, exhibiting its platelet-aggregation inhibitory action. When heparin was added to PRP simultaneously with PGI_2 and incubated for 10 minutes before the addition of ADP, the inhibitory pattern of PGI_2 was partially blocked, and the ADP-induced aggregation was now 52 percent. This indicated that heparin blocked the PGI_2-induced inhibitory activity not only in the isolated system where PGI_2 was incubated with heparin, but also in the PRP system in which both agents were added simultaneously and incubated with platelets.

Heparin has been reported to bind irreversibly to platelets (*19*). A study therefore was made to determine whether heparin exposed to platelets and subsequently exposed to PGI_2 would continue to exhibit a time-related progressive neutralization of PGI_2 as observed when these two agents were incubated together prior to their addition to platelets. For this experiment, lower concentrations of heparin (10, 5, and 2.5 unit/ml) were incubated with platelets in plasma for 10 minutes. The PGI_2 was then added, and the mixture was incubated further. At 0, 5, and 10 minutes after the addition of PGI_2, ADP was added. Controls consisted of replacing heparin and PGI_2 with buffer. Results of this set of experiments with 10 units per milliliter of heparin are shown in Table 2. The same pattern of neutralization was seen with lower concentrations of heparin. Platelets incubated with buffer or heparin in the absence of PGI_2 showed a slight decrease in aggregation as the incubation time increased (Table 2, lines 1 and 2). When platelets were incubated with PGI_2 in the absence of heparin, there was a progressive increase in inhibition that was directly proportional to the time the platelet-PGI_2 mixture was incubated (from 44

Table 2. Neutralization of PGI_2 by heparin first incubated with PRP. A 0.2-ml sample of PRP was incubated with 0.1 ml (10 unit/ml) of heparin for 10 minutes; 0.1 ml (0.005 μM) of PGI_2 was then added to this mixture. For controls, buffer replaced PGI_2 or heparin, or both. These mixtures were then incubated further for up to 10 minutes; aggregation was obtained by addition of 0.1 ml ($10^{-5} M$) of ADP at 0, 5, and 10 minutes. Percentage of aggregation shown is the percentage of transmission (arbitrary units) recorded 1 minute after addition of ADP.

Incubation mixture				Percent of aggregation at		
Buffer	PGI_2	Heparin	ADP	0 min	5 min	10 min
+	−	−	+	62	50	50
+	−	+	+	75	60	60
+	+	−	+	44	24	20
−	+	+	+	58	42	46

to 20 percent; Table 2, line 3). When platelets were first incubated with heparin and then treated with PGI_2, the inhibitory action of PGI_2 was decreased at 0, 5, and 10 minutes, compared to the effects of PGI_2 in the absence of heparin (Table 2, lines 4 and 3). These findings indicate neutralization or blocking of the PGI_2 inhibitory pattern by heparin. The reaction, however, does not exhibit a time-related progressive neutralization of PGI_2 by heparin, as observed when both agents were incubated together before addition to platelets (Fig. 2).

It is not clear at this time why neutralization of PGI_2 by heparin occurs in a time-dependent manner when the two are mixed in the absence of PRP and in a nontime-dependent fashion when PRP is present although several explanations can be offered. When heparin is incubated with PRP, its effective concentration may decrease as a result of binding with the several antiheparin factors present on the platelet and in the plasma. Therefore, less heparin would be available to block the inhibitory activity of PGI_2. Further, effects of PGI_2 become more marked as it is incubated with platelets. It is possible also that platelet-bound heparin has a decreased affinity to interact with PGI_2, compared to heparin directly added to PGI_2. Furthermore, it is conceivable that both PGI_2 and heparin might have separate binding sites on platelets. When PGI_2 is added to platelets that had already been incubated with heparin, it is partially neutralized by available heparin while it can still demonstrate a decreased inhibitory activity by acting with its specific sites on platelets.

Heparin did not appear to lose its anticoagulant activity when incubated with PGI_2 for 10 minutes. When the mixture of heparin and PGI_2 was tested at the end of the 10-minute incubation interval for effects on the thrombin-fibrinogen clotting system (Table 3), heparin inhibition of clotting was not diminished.

Heparin also inhibited the increase of platelet cyclic AMP usually mediated by PGI_2. The PGI_2 was incubated with buffer or with heparin for 10 minutes and then added to PRP and these mixtures were evaluated for their effects on platelet cyclic AMP levels. The PGI_2 alone (Fig. 3) increased platelet cyclic AMP to 31.5 pmole, but when added as a mixture with heparin, the effect was markedly diminished since the level fell to 7.8 pmole. Thus, heparin inhibited the elevation of platelet levels of cyclic AMP normally produced by PGI_2.

These studies indicate that heparin interacts directly with PGI_2 and blocks the inhibitory activity of this prostacyclin on platelet aggregation. The study also suggests that although in the heparin-PGI_2 interaction product PGI_2 loses its inhibitory activity on platelets, the anticoagulant activity of heparin remains intact. These observations suggest that heparin may produce or at least contribute to hyperaggregation of platelets and thrombocytopenia in certain patients receiving this anticoagulant but maintain its clinically measurable anticoagulant effects.

Different patients may have varying amounts of circulating agents capable of inducing platelet aggregation. It may be that only in patients with higher levels of such agents would treatment with heparin produce thrombocytopenia. Further, the exact mechanisms controlling PGI_2 production and release from endothelium are not known, nor is it known whether different patients may show different responses to the stimuli that enhance PGI_2 synthesis and release. Until these latter facts are determined, the full role of heparin in induction of thrombocytopenia cannot be established.

Table 3. Effects of PGI_2 and heparin on thrombin clotting time. A 1:1 mixture of PGI_2 and heparin (0.8 unit/ml) was incubated for 10 minutes at 37°C. As a control, buffer alone or with PGI_2 or heparin was incubated. A 0.1-ml sample of each of these mixtures was added to 0.2 ml of normal human plasma, and the clotting time was recorded after the addition of 0.2 ml (10 units) of thrombin. Results represent the means of three studies, performed in duplicate.

Incubation mixture	Clotting time (seconds)
Buffer	17.7
PGI_2* + buffer	16.5
PGI_2† + buffer	18.5
Heparin + buffer	42.5
PGI_2* + heparin	40.5
PGI_2† + heparin	43.5

*PGI_2 concentration was 0.02 μM. †PGI_2 concentration was 2.7 μM.

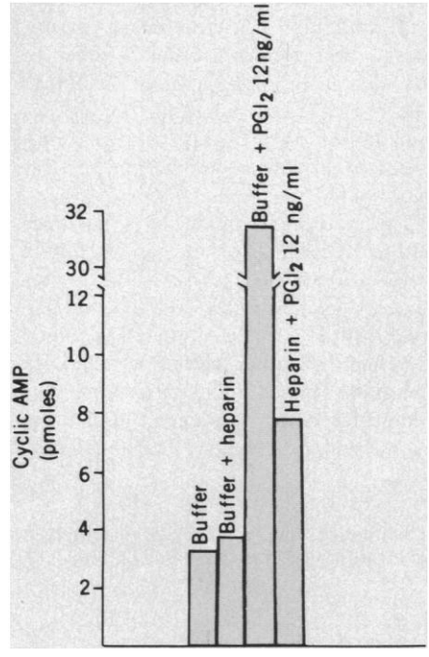

Fig. 3. Effect of heparin on PGI_2-mediated cyclic AMP synthesis in platelets. A 1:1 mixture of PGI_2 and heparin was incubated for 10 minutes at 37°C. In the mixture, the final concentration of PGI_2 was 0.04 μM (12 ng/ml); that of heparin was 10 unit/ml. For controls, buffer was incubated alone or with PGI_2 or heparin. A 3.0-ml sample of the test or control mixtures was added to 3.0 ml of PRP, and the cyclic AMP level was determined. (Each bar graph represents the result of at least three experiments.)

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