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Platelet-Vessel Wall Interaction: Role of Blood Clotting [and Discussion]

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Platelet – vessel wall interaction: role of blood clotting

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Vascular damage initiates not only the adhesion and aggregation of blood platelets but also coagulation, which is of mixed (intrinsic and extrinsic) origin. Evidence is presented that thrombin, generated as a result of the injury, is a prerequisite for platelet aggregation.

Platelets, after activation, in their turn promote coagulation. Prostaglandin I_2 (PGI_2 or prostacyclin) inhibits coagulation induced by damaged vascular tissue. This effect of PGI_2 is mediated by the inhibition of platelets in their participation in the generation of factor X_a and thrombin. Dietary cod liver oil, by changing plasma coagulability, decreases the procoagulation activity of vessel walls, and arterial thrombosis. Another fish oil with similar effects on plasma coagulability and some other haemostatic parameters does not modify vessel wall-induced clotting, nor does it significantly lower arterial thrombosis tendency; this indicates the physiological relevance of vessel wall-induced clotting in arterial thrombus formation. Some evidence is also given for the importance of vessel wall-induced clotting in primary haemostasis.

1. Introduction

The major event triggering haemostasis and thrombosis is disruption of vascular endothelium. Exposure of the blood to subendothelium results in the simultaneous occurrence of two closely interrelated processes: platelet activation and blood clotting. Platelet activation may lead to the formation of a fragile platelet thrombus by a process reviewed by Mustard & Packham (1970), Weiss (1975), and many others. In brief, circulating platelets adhere to subendothelial tissue – collagen, microfibrils, basement membrane – which may become exposed to the blood after vessel trauma, rupture of an atherosclerotic plaque, etc. Adhered platelets release some of their constituents, such as adenine nucleotides, serotonin, Ca^{2+} and adrenalin. Adenosine diphosphate (ADP), released by this reaction, causes passing platelets to aggregate and stick to the adhered ones, thus forming a mural platelet thrombus which, being unstable, is easily embolized. The platelet-release reaction is also induced by platelet aggregation; so the formation of mural and circulating platelet thrombi is a self-propagating process.

Since vascular tissue has thromboplastic activity (Nemerson & Pitlick 1972), vessel wall damage is likely to trigger extrinsic clotting. Moreover, subendothelial collagen, while interacting with one of the contact factors (factor XII) (Niewiarowski *et al.* 1965; Wilner *et al.* 1968) or with platelets (Walsh 1972*a*), triggers the intrinsic coagulation system, which is also initiated by platelets *per se* when activated by small amounts of ADP (Walsh 1972*b*). Coagulation is accelerated by platelet factor 3 (PF_3), a phospholipoprotein entity that is normally inactive (Fantl & Ward 1958) but which becomes available upon platelet activation (Sixma & Nijessen 1970; Joist *et al.* 1974).

The contribution of coagulation to arterial thrombus formation has long been thought to

be of secondary importance, being confined to reinforcement by fibrin of the fragile white platelet thrombus. However, as will be demonstrated below, there is evidence that coagulation is of primary importance here, because thrombin formation appeared to be necessary for the platelet response to vessel wall damage *in vitro*.

2. Measurement of vessel wall-induced clotting

Rats are bled under ether anaesthesia by puncturing the abdominal aorta. The blood is collected in citrate; platelet-rich plasma (p.r.p.) and platelet-poor plasma (p.p.p.) are prepared by differential centrifugation. The aortas are rapidly removed, cleaned of adhering

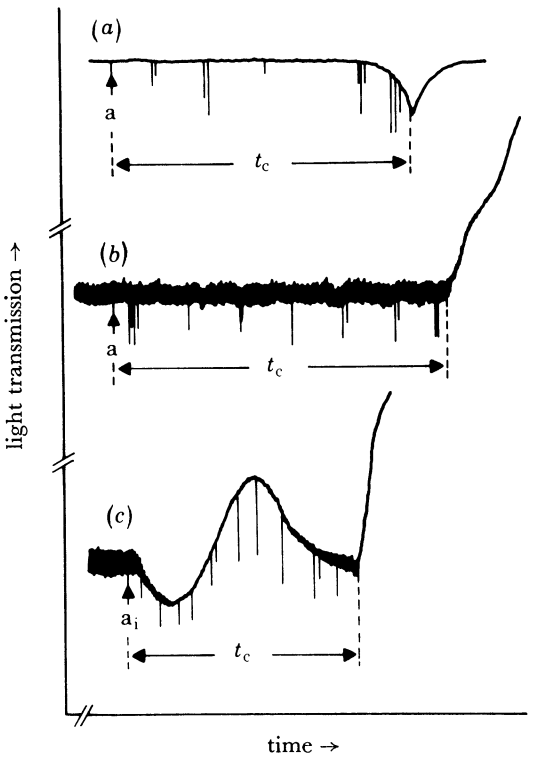

Figure 1. Clotting-induced changes in light transmission in platelet-poor plasma (*a*) and platelet-rich plasma preceded (*c*) or not (*b*) by platelet shape change and aggregation. Abbreviations: a, aorta; a_i, aorta pretreated with indomethacin; t_c, clotting time.

tissue, opened longitudinally and kept in an ice-cold Krebs–Henseleit (K.H.) buffer. P.r.p. or p.p.p. (50 µl) and 450 µl Ca^{2+}-containing saline ($CaCl_2$, 0.65 µmol/l, in NaCl, 0.154 mol/l) are placed into the cuvette of an aggregometer. The temperature is maintained at 37.5 °C and the stirring speed is 600–700 rev/min. After 3 min, a small piece of tissue, 3 mm in diameter, is punched out of the aorta and transferred into the cuvette, where light transmission is recorded continuously. Fibrin formation is indicated by a decrease in light transmission, which is very clearly observed in p.p.p. (figure 1*a*). This decrease is followed by a rapid increase when the fibrin fibres become twisted around the stirring bar. When the vascular tissue passes the light beam, the tracing shows spikes. In p.r.p., fibrin formation is not always clearly visible, owing to the high turbidity of the solution. However, the subsequent increase in trans-

mission is more pronounced than in p.p.p., because platelets are trapped in the fibrin strands twisting around the stirring bar (figure 1*b*). The time lapse (in seconds) between tissue addition and the moment of clotting is called the clotting time, t_c, which, for statistical reasons, is indicated by the clotting index $S = 1000/t_c$. The higher S, the higher is the clotting tendency of the plasma. So far, vessel wall-induced clotting has been observed with material from rabbits, rats, dogs and man. Most of the experiments to be described here have been performed with material from rats.

The tissue is punched out of a blood vessel; therefore our model may be more related to haemostasis than to thrombosis since in the latter process vascular damage is more superficial. However, the basic processes in haemostasis and thrombosis may be similar; therefore our findings may be relevant for both haemostasis and thrombosis.

3. Tentative characterization of the clotting process initiated by damaged vascular tissue

The process of blood clotting can be activated via two different routes (figure 2). (For a review see Suttie & Jackson (1977).) In the intrinsic pathway, which can be measured by the a.p.t.t. test (activated partial thromboplastin time), only blood-borne clotting factors are involved. Extrinsic clotting is initiated by a tissue thromboplastin and, moreover needs clotting factor VII. It can be measured by the p.t. test (prothrombin time).

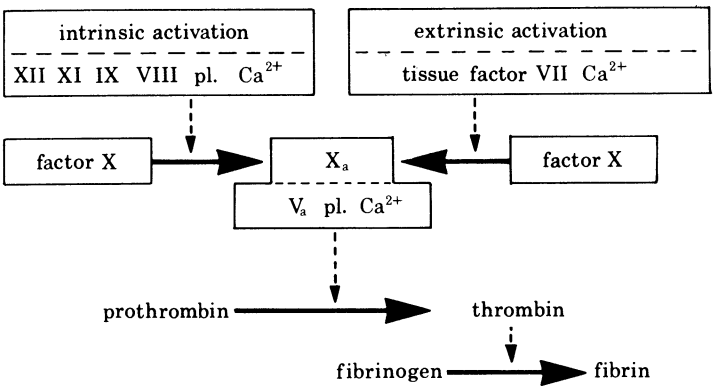

Figure 2. Simplified diagram of coagulation pathways; pl., platelets or phospholipids.

So far, only preliminary investigations have been carried out on the vessel wall-induced clotting process. When compared with rat-brain thromboplastin, damaged tissue of rat aorta induced similar clotting indices in human VII-deficient plasma, which were about 60% lower than in a standard plasma (figure 3). When human vascular tissue was used (obtained from surgical patients), the clotting response in human VII-deficient plasma was about 40% lower than that obtained with a standard plasma. Both results indicate that the vessel wall activates the extrinsic clotting mechanism. This is in line with the fairly well documented tissue-factor activity of the vessel wall (for review see Nemerson & Pitlick 1972).

This finding was confirmed in preliminary experiments with a rabbit-raised fibrinogen-free and complement-free antiserum against human brain thromboplastin (prepared and provided by Dr K. van Ginkel, Amsterdam), which was shown to be ineffective in the a.p.t.t. test.

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Pieces of vascular tissue from rat or man were incubated in 50 µl antithromboplastin or control serum, diluted with 450 µl Tris buffer (10 mmol/l) in saline (pH 7.3), containing Ca^{2+} at 4 mmol/l, in a stirred cuvette at 700 rev/min and 37 °C. After incubation for 3 and 15 min, 50 µl portions of the incubate were transferred into an aggregometer cuvette, containing 450 µl Tris–saline buffer with Ca^{2+} (4 mmol/l) and 50 µl standard rat or human platelet-

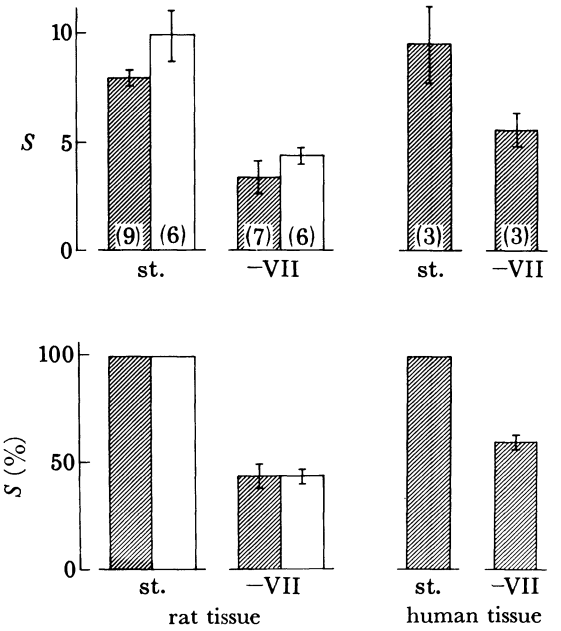

FIGURE 3. Effect of VII-deficient plasma (human) on clotting induced by damaged vascular tissue (shaded bars) and rat brain thromboplastin (open bars). Upper row, clotting indicated by the mean clotting index $S \pm$ s.e.m. (standard error of the mean); lower row, clotting responses calculated as % ± s.e.m. compared with standard plasma (100 %). St., standard plasma; −VII, VII-deficient plasma.

free plasma, which had been preincubated for 3 min at 37.5 °C at a stirring rate of 700 rev/min. The clotting response was recorded and calculated as the clotting index, S (§2).

In figure 4, the open bars represent the clotting responses of the control incubate. The shaded bars refer to the clotting reaction upon addition of the antithromboplastin incubate. The difference between these responses as a percentage of the control S is indicated by the black bars and it can be seen that the clotting potency of rat and human tissue is greatly diminished (but not blocked) upon preincubation with antithromboplastin antibodies. The fact that neither the VII-deficient plasma nor the thromboplastin antibodies are able to prevent vessel wall-induced clotting completely suggests that in this clotting process an intrinsic component is also involved. This is supported by studies of VIII-, IX-, XI and XII-deficient plasmas (human), which showed a lower clotting response with rat aortic tissue than a human standard plasma. The combined character of vessel wall-induced clotting (intrinsic and extrinsic) is also underlined by the significant correlation existing between vessel wall-induced clotting and p.t. as well as a.p.t.t., as was observed in a study of dietary fish-oil induced changes in vessel wall-induced clotting (see §9). However, more experiments are needed for a further analysis of this clotting process.

4. Vessel wall-induced platelet reactions: role of thromboxane A_2 and ADP

The clotting of p.r.p., brought about by a piece of vascular tissue producing little or no prostacyclin, is preceded by platelet shape change and aggregation (figure 1*c*). The time course of these platelet reactions very much resembles that of collagen-induced aggregation, which is known to be mediated by ADP (Hovig & Holmsen 1963) and by thromboxane A_2, (TXA_2) (Hamberg *et al.* 1975) which are derived from activated blood platelets and which can be inhibited by aspirin (O'Brien 1968; Weiss *et al.* 1968; Zucker & Peterson 1970).

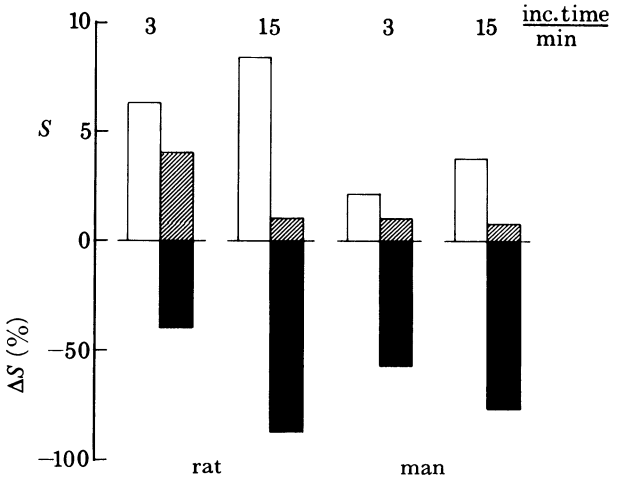

Figure 4. Effect of an antithromboplastin antiserum on the clotting index (*S*) of rat and human tissue. Open bars, tissue incubated with control serum; shaded bars, tissue incubated with antiserum; solid bars, difference in clotting response occurring upon tissue incubating in control and antiserum respectively (ΔS).

To investigate the role of platelet ADP and TXA_2 in the aggregation of p.r.p. after exposure to damaged vascular tissue, p.r.p. was preincubated with aspirin (0.55 mmol/l in saline) or with saline for 1 h at room temperature. Subsequently, aggregation was measured in 50 µl p.r.p. diluted with 440 µl Ca^{2+}-containing saline (Payton Dual Channel aggregometer), on addition of one piece of indomethacin-treated vascular tissue (with 10 µl saline), 10 µl ADP (0.25 µmol/l, final concentration), 10 µl of a suspension of collagen in saline (5.6 µg protein/ml, final concentration) or 10 µl thrombin (0.09 N.I.H. units/ml, final concentration).

The results of this study are shown in figure 5*a*, from which it is evident that the aggregation induced by injured vascular tissue and by thrombin is not significantly affected by aspirin treatment of the p.r.p. The collagen- and ADP-induced aggregation was significantly impaired, thus showing the effectiveness of the aspirin dose used to block platelet TXA_2 production. These results strongly suggest that vessel wall-induced aggregation is not primarily mediated by TXA_2 and/or ADP.

However, it has been shown repeatedly that the aspirin effect can be overcome by simply increasing the trigger strength, thereby inducing the release of ADP independently of platelet thromboxane production (Zucker & Peterson 1970; Fukami *et al.* 1976). Therefore, the aspirin experiment was repeated with p.r.p. obtained from Fawn Hooded (FH) rats. This strain of rats has a hereditary defect of the platelet release reaction: their dense granules, which in

normal animals contain ATP, ADP, Ca^{2+}, serotonin, pyrophosphate and antiplasmin (Holmsen 1978), seem to be empty. This defect is thought to be the cause of the bleeding tendency observed in these animals, because the collagen-induced aggregation is greatly impaired (Raymond & Dodds 1975; Tschopp & Zucker 1972). However, collagen-induced platelet TXA_2 production is normal in FH rats, judging from the production of a TXA_2 metabolite, malondialdehyde (MDA), upon supramaximal triggering with collagen (FH, 1.33 ± 0.048; Wistar strain, 1.30 ± 0.069 nmol MDA/10^9 platelets; $n = 9$).

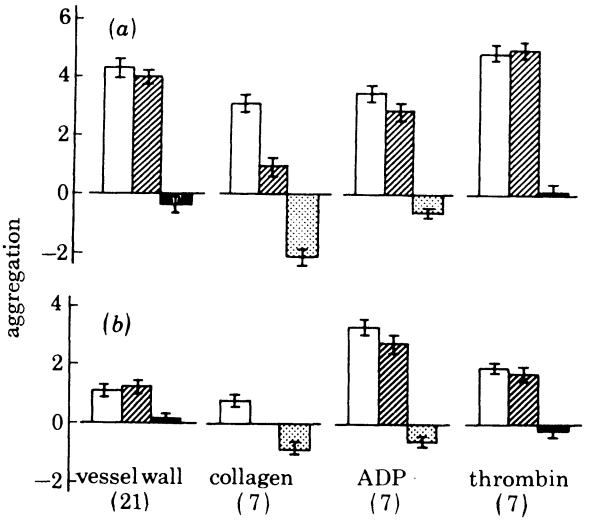

FIGURE 5. Aggregation (arbitrary units, ±s.e.m.) of normal (open bars) and aspirin-treated (hatched bars) p.r.p. induced by different stimuli. (*a*) Wistar rats; (*b*) Fawn Hooded rats. Solid black bars, difference between normal and aspirin-treated p.r.p. not significant ($p_2 > 0.05$); stippled bars, difference significant ($p_2 < 0.01$); numbers in parentheses, *n*.

As shown in figure 5*b*, the results obtained with FH p.r.p. are identical to those obtained with Wistar rats. Preincubation of p.r.p. with an aspirin dose large enough to block the collagen-induced aggregation completely and inhibit the ADP-induced aggregation significantly did not modify vessel-wall and thrombin-induced aggregation.

The role of released ADP was further investigated by measuring the aggregation of p.r.p. induced by a piece of indomethacin-treated rat aorta in the presence and absence of the ADP dephosphorylating enzyme apyrase (Ardlie *et al.* 1971) and the ADP transphosphorylating system creatine phosphate and creatine phosphokinase (Izrael *et al.* 1974). These ADP scavengers appeared to inhibit platelet aggregation only in doses about double those needed to block a comparable degree of aggregation induced by added ADP.

These results demonstrate that neither TXA_2 nor ADP plays a primary role in the aggregation of platelets induced by damaged vascular tissue.

5. VESSEL WALL-INDUCED PLATELET REACTIONS: ROLE OF THROMBIN

As shown in figure 5*a*, *b*, the aggregation induced by damaged vascular tissue closely resembles that of thrombin as far as the effect of aspirin is concerned. Since the p.r.p.–vessel-wall interaction triggers a clotting response, the preceding platelet reactions may be mediated

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by thrombin formed during this clotting response. We tested this hypothesis by measuring vessel wall-induced aggregation and clotting of p.r.p. in the presence of increasing amounts of hirudin (from Pentapharm, Basle, Switzerland), which is a specific thrombin-inactivating polypeptide and does not modify platelet aggregation induced by ADP. It appeared that this substance very effectively blocked platelet aggregation when added to p.r.p. in doses only slightly effective in lowering the clotting response (figure 6).

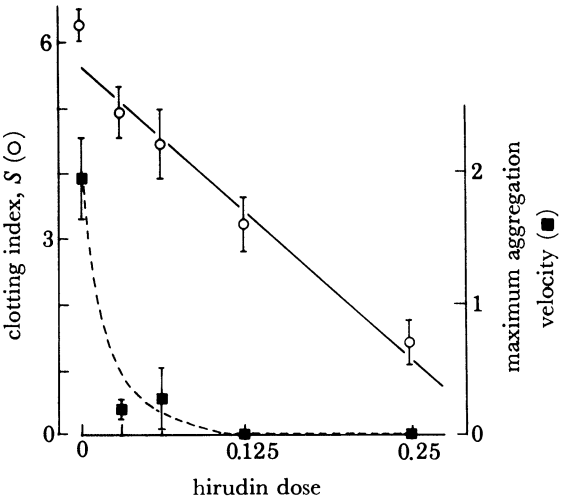

FIGURE 6. Effect of hirudin (final concentrations in antithrombin units per millilitre) on clotting (○) and platelet aggregation (■) induced by damaged vascular tissue; mean values ±s.e.m.; $n = 5$.

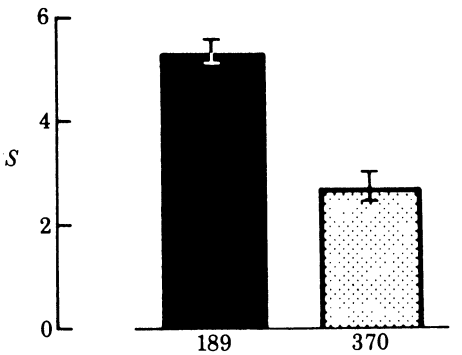

FIGURE 7. Effect of blood platelets on vessel wall-induced clotting ($S \pm$ s.e.m.). Figures below bars are clotting times (seconds) calculated from mean S values; $n = 28$. Solid bar, clotting occurring in p.r.p.; stippled bar, clotting occurring in p.p.p.

It is therefore concluded that platelet aggregation after exposure to damaged vascular tissue is mediated by thrombin, generated during vessel wall-induced clotting. Since ADP scavengers had also some inhibitory effect on vessel wall-induced platelet reactions, a supporting role of ADP, released from erythrocytes, adherent platelets and/or from non-adherent platelets activated by the generated thrombin, is probable (Packham *et al.* 1973).

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6. Role of blood platelets in vessel wall-induced clotting

When vessel wall-induced clotting in p.r.p. and p.p.p. was compared, it appeared that the clotting response in p.r.p. is significantly greater than that in p.p.p. (figure 7; $p_2 < 0.01$, Student's t-test). Although the pieces of vascular tissue used provide a thrombogenic surface, especially along their edges, platelets not in direct contact with the vessel wall are not activated, as can be concluded from the absence of platelet shape change and aggregation (figure 1*b*).

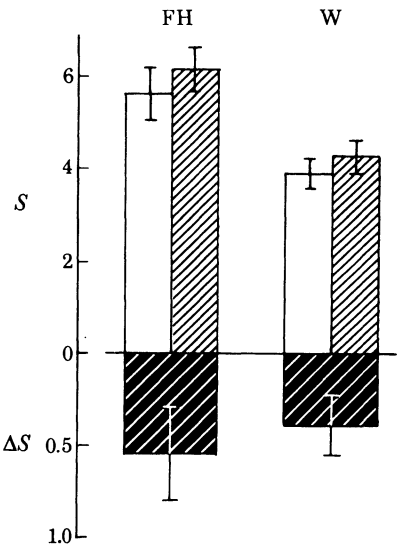

Figure 8. Effect of ADP-induced platelet aggregation on vessel wall-induced clotting ($S \pm$ s.e.m.) of p.r.p. Experiments carried out with material from Fawn Hooded rats (FH) and Wistar rats (W); $n = 12$. Open bars, clotting of p.r.p. without ADP added; upper hatched bars, clotting of p.r.p. when aggregation is induced by adding ADP; lower hatched bars, difference in clotting ($\Delta S \pm$ s.e.m.) resulting from ADP-induced aggregation.

This would indicate that, for their clot-promoting effect, platelets need not become activated. However, it is likely that the adhered and thereby activated platelets are responsible for the clot-promoting effect observed. When, immediately upon tissue addition, aggregation is triggered by adding ADP 0.35 μmol/l, final concentration), coagulation is significantly enhanced ($p_2 < 0.05$) (figure 8). Since ADP in itself has never been shown to affect blood clotting, this strongly suggests that activated platelets have a stronger clot-promoting effect than non-activated ones. As is also evident from figure 8, vessel wall-induced clotting in FH rats (see § 4) is significantly ($p_2 < 0.01$, Student's two-samples test) higher than in normal Wistar rats. This has been confirmed in later experiments and requires further investigation.

7. Effect of vascular prostacyclin on vessel wall-induced clotting

Vascular tissue produces prostacyclin (PGI_2), which is a very active inhibitor of platelet activation (Moncada *et al.* 1976) and may therefore be expected to inhibit the participation of blood platelets in vessel wall-induced clotting. When vascular prostacyclin formation is blocked by pretreatment of the tissue with indomethacin, aspirin or tranylcypromine, the clotting response is enhanced; moreover, it is preceded by a generalized activation of the platelets in the cuvette as indicated by their shape change and aggregation. Indeed, this finding suggests that vascular prostacyclin, by diminishing platelet activation, inhibits vessel

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wall-induced clotting. However, an alternative explanation may be that the compounds used to prevent prostacyclin generation affect vessel wall induced clotting in a direct, prostacyclin-independent way. We therefore measured vessel wall-induced clotting with material taken from arachidonic acid (AA) deficient animals. Vascular tissue of these animals produces only very small amounts of prostacyclin (Hornstra *et al.* 1978). Consequently, if PGI_2 does indeed play a regulating role, vessel wall-induced clotting might be expected to be enhanced in AA

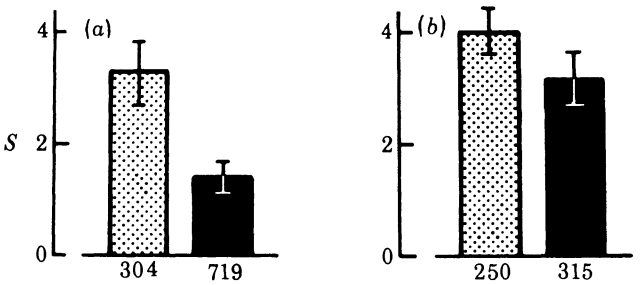

FIGURE 9. Vessel wall-induced clotting ($S \pm$ s.e.m.) observed with material obtained from AA-deficient (stippled bars) and control (solid black bars) animals. (*a*) Untreated tissue incubated in autologous p.r.p. ($n = 10$); (*b*) indomethacin-treated tissue, incubated in autologous p.r.p. ($n = 12$). Figures below bars are clotting times (seconds) calculated from mean S values.

deficiency. This indeed appeared to be so: clotting induced by AA-deficient tissue is significantly higher than with control material (figure 9*a*). Only in the AA-deficient group, clotting was preceeded by platelet shape change and aggregation.

When the tissue was pretreated with indomethacin, thereby preventing any role of vascular prostacyclin, the difference between both groups was greatly reduced (figure 9*b*). These and other findings (Hornstra & Hemker 1979) demonstrate that vascular prostacyclin inhibits vessel wall-induced clotting.

8. Mechanism by which PGI_2 inhibits vessel wall-induced clotting

S. Bunting & S. Moncada (personal communication) and Ts'ao *et al.* (1979) demonstrate that clotting tests performed in the absence of platelets are not modified by PGI_2. Moreover, vessel wall-induced clotting in p.p.p. is not affected by pretreatment of the tissue with indomethacin. Only in the presence of platelets has prostacyclin been demonstrated to inhibit clotting. Therefore, any effect of prostacyclin on vessel wall-induced clotting is probably mediated by its effect on blood platelets.

Platelets contribute to blood coagulation in various ways (figure 10): when activated with collagen or ADP they initiate the intrinsic pathway of coagulation (Walsh 1974). Moreover, they contribute to the intrinsic activation of factor X and the conversion of prothrombin into thrombin. (For a review see Zwaal (1978)). This latter activity is known as platelet factor 3 (PF_3) and has been shown to reflect procoagulant phospholipids (mainly phosphatidyl serine) becoming available at the platelet surface. For the expression of PF_3 activity, intact platelets must become activated and it appeared that of all physiologically relevant platelet activators only a combination of collagen and thrombin is able to make these activities available (Zwaal *et al.* 1980). Recently the same was shown to hold for the role of platelets in the activation of factor X

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(G. van Dieijen, personal communication). Since this combined trigger is likely to be present at the site of a vascular injury (collagen from the subendothelium and thrombin as a result of vessel wall-induced clotting), it is conceivable that the effect of blood platelets on vessel wall-induced clotting is at least partly due to their stimulation of X_a-generation, prothrombin conversion, or both. Consequently, PGI_2 may inhibit vessel wall induced clotting by inhibiting these platelet functions. This is currently under investigation with the use of the following

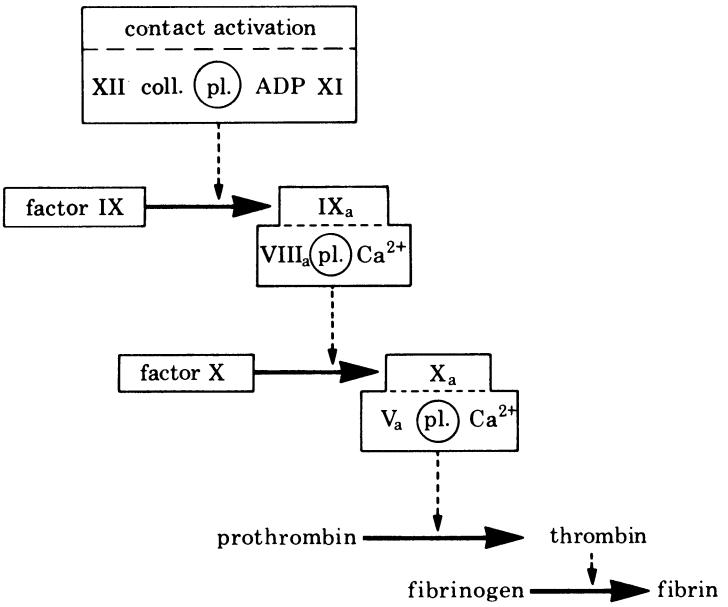

FIGURE 10. Simplified diagram to indicate the participation of platelets (pl.) in the various stages of coagulation.

assay system. A mixture of the appropriate amounts of Ca^{2+} and of the various purified clotting factors is incubated with washed platelets preactivated with collagen and thrombin in the presence of different amounts of PGI_2. After a given incubation time the reaction is interrupted and the amount of X_a or thrombin formed is measured spectrophotometrically, by using X_a or thrombin-specific chromogenic substrates and standard curves obtained with purified X_a and thrombin (Rosing *et al.* 1980; van Dieijen *et al.* 1981).

When no platelets are present, or when the platelets are not properly activated, X_a generation is hardly measurable. However, the presence of activated platelets greatly accelerates X_a formation, and preliminary results (figure 11) clearly indicate that this process is inhibited by PGI_2 in a dose-dependent way. No inhibition is observed when the procoagulant phospholipids are added as lipid vesicles or when lysed platelets are used as a source of these phospholipids. This demonstrates that prostacyclin does not interfere with X_a formation as such, but specifically inhibits the process by which collagen and thrombin stimulate platelets to expose procoagulant phospholipids.

Essentially similar, although quantitatively different, results were obtained for the participation of platelets in the generation of thrombin. As shown in figure 11, a rather high dose of PGI_2 is required to inhibit those platelet-procoagulant activities; moreover the inhibition is only partial. This partial inhibition is also observed, although to a lesser extent, when the

aggregation of blood platelets and their ATP release is measured in response to a mixture of collagen and thrombin. Although each trigger can on its own be effectively blocked by PGI_2, their combined action cannot be inhibited completely. It is tempting to speculate that the rest-activity is due to platelets that have adhered to the collagen, despite the high PGI_2 concentration. From our preliminary data it seems that platelet thrombotic functions (aggregation

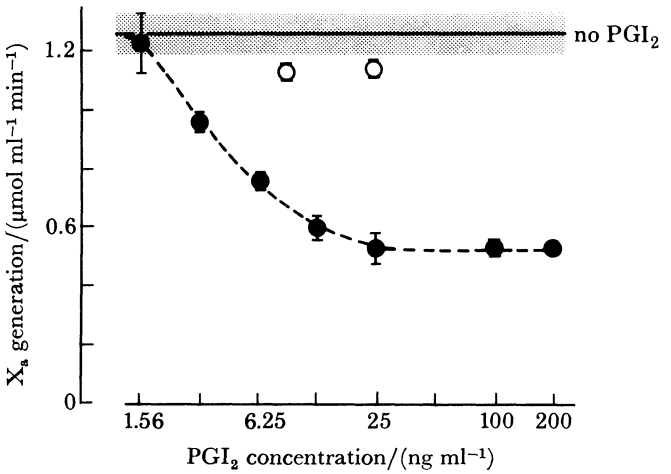

FIGURE 11. Effect of prostacyclin (PGI_2) on the participation of washed platelets (2.7×10^7/ml), activated with a mixture of collagen and thrombin, in the generation of factor X_a. Means of 2–6 determinations $\pm$s.e.m. Open circles refer to measurements performed with platelet lysates, diluted to obtain about the same X_a generation as the platelet preparation under investigation (value without PGI_2: 1.12 ± 0.02 μmol X_a ml^{-1} min^{-1}).

and release) are about 10 times more sensitive to inhibition by prostacyclin than the platelet procoagulant activities under investigation. Further experiments are in progress to explore these phenomena.

9. EFFECT OF DIETARY FISH OIL ON VESSEL WALL-INDUCED CLOTTING

From the experiments with AA-deficient animals (see §7) it appeared that vascular prostacyclin inhibits vessel wall-induced clotting. As with AA deficiency, feeding fish oil also results in a diminished production of vascular prostacyclin (Hornstra & Hemker 1979; Ten Hoor *et al.* 1980). Moreover, feeding fish oils to rats does not result in the formation of prostaglandin I_3 (Hornstra *et al.* 1981). Because of this low production of PGI, vessel wall-induced clotting may be expected to be enhanced by fish oil feeding. However, as has been demonstrated before for FH rats (Hornstra & Hemker 1979), feeding cod liver oil results in a significant inhibition of this vascular clotting response, which appeared to be due to some plasma condition. This cod liver oil effect has been confirmed with Wistar rats; it is not produced by fish oils in general since feeding another fish oil did not result in a lower vessel wall-induced clotting response (figure 12). Because cod liver oil and the other fish oil lowered platelet and vascular prostaglandin production to about the same extent (Hornstra 1981), vascular prostacyclin is unlikely to be involved. Moreover, the cod liver oil effect is likely to act through the plasma, excluding any other vascular factor. We therefore measured plasma coagulability in animals fed with fish oil and compared it with that of plasma obtained from animals fed

either a commercial stock diet or a diet enriched with sunflower seed oil. To investigate the intrinsic pathway of coagulation, a.p.t.ts were measured in platelet-free plasma (p.f.p.) prepared by high-speed contrifugation (16000 *g* for 20 min) of p.p.p. The test was performed in p.f.p., diluted to 20% with a Tris buffer (10 mmol/l in saline) at pH 7.35. The activation mixture consisted of inositin, 50 mg/ml (0.2 ml); kaolin, 100 mg; and Tris–saline buffer (19.8 ml). Clotting was measured in an aggregometer (Payton Dual Channel) at 37.5 °C in siliconized glass tubes. Transmitted light was recorded continuously while the reaction mixture

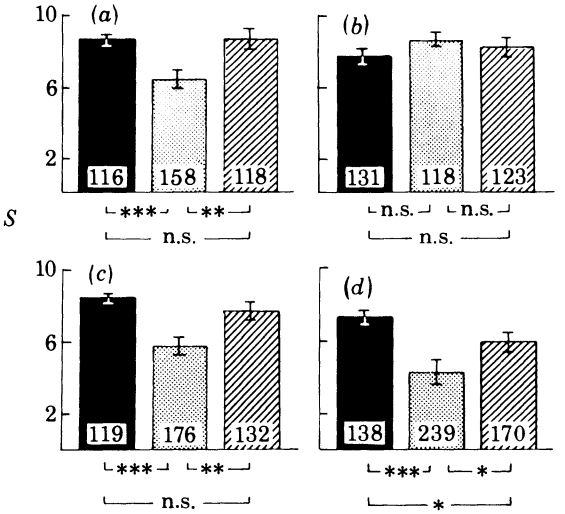

FIGURE 12. Vessel wall-induced clotting ($S \pm$ s.e.m., $n = 10$) measured with vascular tissue, p.r.p. and p.p.p. of rats fed on diets containing 50% of its digestible energy as sunflower seed oil (black bars), cod liver oil (shaded bars), and another fish oil (hatched bars). Figures in bars represent clotting times (seconds) calculated from mean S values. (*a*) Vascular tissue in autologous p.r.p.; (*b*) vascular tissue in p.r.p. of stock animals; (*c*) vascular tissue of stock animals in different p.r.ps; (*d*) vascular tissue of stock animals in various p.f.ps. Significance levels: n.s., $p_2 > 0.10$; *, $0.05 < p_2 < 0.10$; **, $0.001 < p_2 < 0.01$; ***, $p_2 < 0.001$.

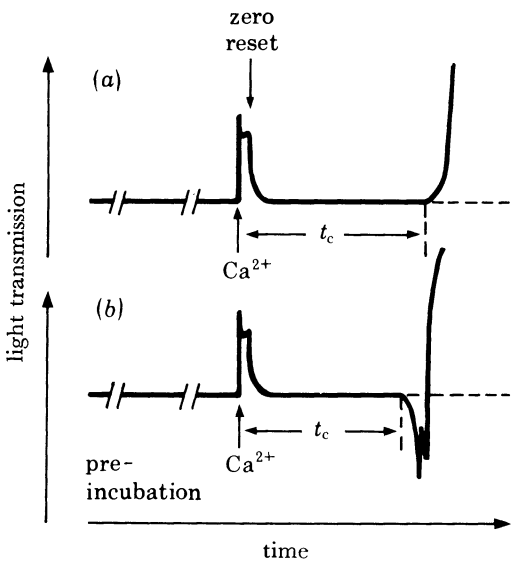

FIGURE 13. Diagram of aggregometer tracings obtained when measuring a.p.t.t. (*a*) and p.t. (*b*) in platelet-free plasma dilutions; t_c, clotting times.

was stirred with a siliconized metal bar at 500 rev/min; 0.1 ml Tris–saline buffer, 0.1 ml plasma dilution and 0.1 ml activation mixture were incubated for 2 min, after which 0.1 ml $CaCl_2$ (0.033 mol/l) was added. Clotting times were calculated from the recorder tracings as indicated in figure 13. Because of skew distribution, these clotting times (t_c, seconds) were converted to the clotting index, $S = 1000/t_c$ (see §2).

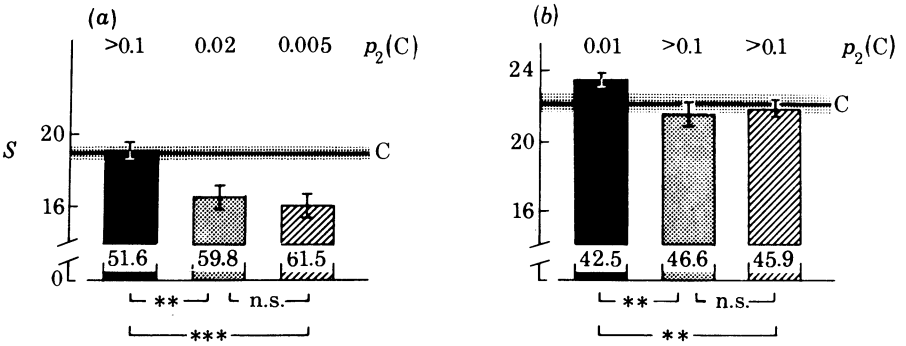

FIGURE 14. Intrinsic (a.p.t.t., (*a*)) and extrinsic (p.t., (*b*)) clotting ($S \pm$ s.e.m., $n = 10$) measured in platelet free plasma dilutions of rats fed on diets containing 50% of their digestible energy as sunflower seed oil (black bars), cod liver oil (shaded bars), and another fish oil (hatched bars). Figures in bars indicate clotting times (seconds) calculated from mean S values. C, Results obtained with plasma of control animals fed a commercial stock diet (heavy line, mean; shaded area, s.e.m.); p_2(C), significance level compared with control group; n.s., $p_2 > 0.10$; **, $0.001 < p_2 < 0.01$; ***, $p_2 < 0.0001$.

Extrinsic clotting was measured with the one-stage p.t. test by using rat-brain thromboplastin prepared according to a procedure described by Owren & Aas (1951). The preparation was diluted with Michaelis buffer to give a clotting time of about 14 s ($S = 71.4$) with undiluted standard rat p.f.p. P.t. measurements were performed on p.f.p., diluted to 20% by volume with the Tris–saline buffer. Plasma dilution (0.15 ml) and 0.15 ml of the rat-brain thromboplastin preparation were incubated for 1 min at 37.5 °C at a stirring rate of 500 rev/min in a siliconized glass cuvette of the Payton Dual Channel aggregometer. Coagulation was triggered by adding 0.15 ml $CaCl_2$ (0.033 mol/l). Clotting times were calculated from the aggregometer tracings as shown in figure 13 and converted to S values as discussed for a.p.t.ts.

As illustrated in figure 14*a*, intrinsic clotting is reduced in both fish oil groups compared with the control group, whereas extrinsic clotting is not significantly altered by fish oil feeding (figure 14*b*). Compared with sunflower seed oil, both intrinsic clotting and extrinsic clotting are reduced in both fish oil groups.

In fact, a significant correlation was observed between vessel wall-induced clotting and plasma extrinsic and intrinsic coagulability (figure 15). This supports the view that vessel wall-induced clotting is of mixed intrinsic and extrinsic origin (see §3). However, these results do not explain the difference between the two fish oils in their effect on vessel wall-induced clotting. This difference therefore needs further investigation.

10. PHYSIOLOGICAL RELEVANCE OF VESSEL WALL-INDUCED CLOTTING

As stated in §2, vessel wall-induced clotting may be important in haemostasis and thrombosis. As far as primary haemostasis is concerned, supporting evidence was obtained from

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experiments with the AA-deficient animals and those fed on cod liver oil. As shown in table 1, both groups of animals show a striking similarity in platelet and vascular prostaglandins and platelet aggregation. However, the bleeding time in the animals fed with cod liver oil is prolonged, whereas in AA-deficient rats it is normal. It might very well be that the enhanced vessel wall-induced clotting observed in AA-deficient animals has effectively counteracted the reduced platelet aggregation after collagen activation, leading to a normal bleeding time,

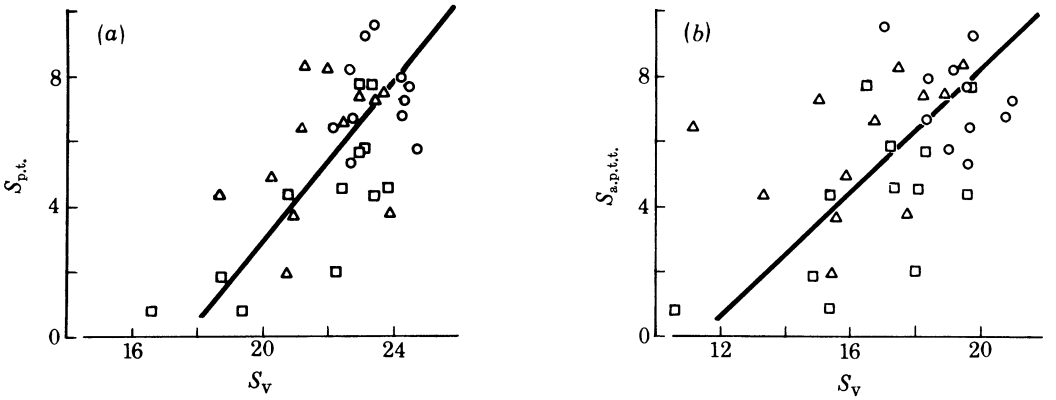

FIGURE 15. Relation between vessel wall-induced clotting (S_v) and extrinsic (p.t., (*a*)) and intrinsic (a.p.t.t., (*b*)) clotting measured in platelet-free plasma of animals fed on diets containing 50 % of its digestible energy as sunflower seed oil (○), cod liver oil (□) or another fish oil (△). (*a*) $y = -21.67 + 1.23x$, $n = 35$, $r = 0.64$, $p < 0.001$; (*b*) $y = -10.73 + 0.95x$, $n = 35$, $r = 0.50$, $0.001 < p < 0.01$.

TABLE 1. COMPARISON BETWEEN COD LIVER OIL FEEDING AND ARACHIDONIC ACID (AA) DEFICIENCY IN THEIR EFFECT ON VARIOUS HAEMOSTATIC PARAMETERS IN RATS

(+, enhanced compared with control animals; −, diminished compared with control animals; ○, not significantly different from control animals.)

parameter	cod liver oil	AA deficiency
platelet and vascular prostaglandins	−	−
aggregation induced by:		
ADP	○	○
collagen	−	−
thrombin	+	+
bleeding time	+	○
vessel wall-induced clotting	−	+

whereas the reduced vessel wall-induced clotting observed in the cod liver oil group may have potentiated the effect of the diminished collagen-induced aggregation, resulting in a prolongation of the bleeding time. In this respect it is also relevant to recall that the lengthening of the bleeding time by aspirin is greatly increased in hypocoagulation (haemophilia, oral anticoagulation, etc.)

Vessel wall-induced clotting is also likely to be important in arterial thrombosis. A first indication was obtained from a study with rats in which different thrombosis tendencies were induced by feeding various dietary fats. After 3 months, arterial thrombosis tendency was measured in 50 % of the animals, whereas the other animals were used for the measurement of vessel wall-induced clotting. It then appeared that both parameters were strikingly corre-

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lated: the higher the vessel wall induced clotting, the higher the tendency towards arterial thrombosis (Hornstra & Hemker 1979).

A second indication of the importance of vessel wall-induced clotting in thrombogenesis is obtained from our fish oil studies. We repeatedly observed that cod liver oil feeding results in a significant lowering of the tendency towards arterial thrombosis, whereas feeding the other fish oil has little effect. This discrepancy between the two fish oils cannot be ascribed to any difference in platelet and vascular prostaglandins, platelet aggregability or plasma coagulability, which are about equal in both groups, as are the bleeding times (Hornstra 1981). Only vessel wall-induced clotting appeared different: it is reduced by feeding with cod liver oil, whereas it is not affected by feeding with the other fish oil (figure 12). It is tempting to speculate that this difference is responsible for the different effects on arterial thrombosis tendency produced by feeding the various fish oils.

As has been pointed out by Loeliger (1976), the preventive effect of oral anticoagulation on myocardial (re)infarction improves with level of anticoagulation. Recently, a well designed study was undertaken to investigate the effect of anticoagulant withdrawal from postmyocardial infarction patients aged 60 years and over. A significantly higher reinfarction rate was observed in the group in which anticoagulant therapy was discontinued. Bleeding complications were only slightly enhanced in the group in which treatment was continued, resulting in a lower overall mortality in this group (de Vries *et al.* 1980). Since vessel wall-induced clotting is closely correlated with plasma coagulability (see §9), these findings in man also support the importance of vessel wall-induced clotting in arterial thrombus formation. Therefore, further investigations into the phenomenon of vessel wall-induced clotting seem worthwhile.

Thanks are due to Mr C. Gardien and Mr J. S. W. Kleinekoort (Unilever Research, Vlaardingen, the Netherlands) for preparing the illustrations (C.G.) and for supervising the rat feeding experiments (J.S.W.K.). Dr G. van Dieijen and Dr E. Bevers cooperated in the measurements of the PGI_2 effects on X_a and thrombin generation (§8). Miss A. D. Muller made the rat brain thromboplastin preparation (§3).

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Discussion

C. R. W. Gray (*Thoracic Unit, Westminster Hospital, London, U.K.*). Dr Hornstra's results do not support the conclusion that endoperoxides pass from platelets to endothelium to increase its production of prostacyclin. The experiment by Begent & Born (1970) demonstrated rapid embolization of platelet thrombi after the iontophoretic application of ADP to small venules was stopped. This could indicate that ADP stimulates the production of endothelial prostacyclin.

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G. Hornstra. In a series of experiments performed under a variety of conditions, we were unable to demonstrate that activated platelets produce endoperoxides for vascular prostacyclin formation (Hornstra *et al.* 1979). As demonstrated in §§7 and 8, prostacyclin inhibits vessel wall-induced clotting by diminishing platelet activation. If ADP did stimulate vascular PGI_2 production, it would also be expected to reduce vessel wall-induced clotting. However, ADP stimulates the vessel wall-induced clotting response (figure 8). This observation does not exclude the suggested interaction between ADP and the vessel wall since the clot-promoting effect of ADP-treated platelets (Walsh 1972*b*) may be more pronounced than the coagulation-inhibiting effect resulting from a possibly stimulated prostacyclin formation. Nevertheless, our findings do not in themselves support the concept that ADP enhances vascular PGI_2 production.

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