Clot-Promoting Effect of Platelet-Vessel Wall Interaction: Influence of Dietary Fats and Relation to Arterial Thrombus Formation in Rats

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Abstract. A small piece of vascular tissue punched from a rat aorta is able to clot plasma. This coagulation process is promoted by blood platelets, especially after their activation. Thrombin, generated by this clotting process, plays a key role in vessel-wall induced platelet activation. Vascular prostacyclin inhibits vessel-wall-induced clotting of platelet-rich plasma, possibly by inhibiting platelet activation. Type and amount of dietary fats were shown to influence vessel-wall-induced clotting via at least four different mechanisms, namely: by modifying vascular prostacyclin formation; by affecting the clotting potency of the vascular tissue per sec; by an effect on some platelet property, probably connected with platelet activation; by influencing a plasma factor.

Each of these mechanisms, as well as the nature of vessel-wall-induced coagulation, requires further investigation.

Introduction

When investigating dietary influences on vascular prostacyclin formation, we noticed that a piece of tissue punched from a rat or rabbit aorta is able to clot platelet-rich plasma (PRP). Since this coagulation process may be important in arterial thrombus formation, we decided to investigate it in more detail.

Materials and Methods

Vessel-Wall-Induced Clotting

Rats are bled under ether anaesthesia by puncturing the abdominal aorta. The blood is collected in citrate; PRP and platelet-poor plasma (PPP) are prepared by differential centrifugation. The aortas are rapidly removed, cleaned from adhering tissue, opened longitudinally and kept in an ice-cold Krebs-Henseleit buffer (KH). 50-μl PRP or PPP and 450-μl Ca^{++}-containing saline (0.5×10^{-6} mol l^{-1} CaCl$_2$ in 0.154 mol l^{-1} NaCl) 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, diameter 3 mm, is punched out of the aorta, transferred into the cuvette while light transmission is recorded continuously. Clotting is indicated by a sudden change in light transmission (fig. 1), following the well-known picture of platelet aggregation, if present. The time lapse 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: $S = 1/t_c \times 1,000$. S is higher, the higher the clotting tendency of the plasma. Initially, measurements were discontinued after a clotting time of 1,200 sec had been reached. The S-value of these measurements was taken as 0.83. In later experiments, the Ca^{++}-content of the saline was increased to 0.65×10^{-6} mol l^{-1}, which prevented the occurrence of these long clotting times.

Vascular Prostacyclin Production in Autologous PRP

A piece of vascular tissue, pretreated or not with indomethacin (2 μg ml^{-1} for at least 1 h), is added to a 1:10 dilution of PRP in Ca^{++}-containing saline in the aggregometer. 30 sec later, adenosine diphosphate (ADP) is added to a final concentration of 0.25×10^{-6} mol l^{-1}; the resulting aggregation is quantified by measuring the tangent to the steepest part of the aggregation slope (V). Due to prostacyclin (PGI$_2$) production, aggregation in the presence of normal vascular tissue is lower than that on addition of a piece of indomethacin-treated aorta. Therefore, the proportional difference in aggregation, induced by ADP in the presence of either a piece of normal aorta or a piece of indomethacin-treated tissue, is taken as a measure of the amount of PGI$_2$ formed.

This technique for measuring vascular PGI$_2$ production is used to include all possible influences due to such physiological factors as plasma prostacyclin inhibitors/stimulators (11, 16, 19, 22, 23, 26) and/or possible differences in platelet prostacyclin sensitivity which are not observed when PGI$_2$ production occurs in buffer and PGI$_2$ measurements are performed by chemical means or by using an indifferent PRP.

Arterial Thrombosis Tendency in vivo

Arterial thrombosis tendency is determined by measuring the obstruction time (OT) of a loop-shaped, polyethylene canula inserted into the abdominal aorta (9).

Platelet Arachidonate Peroxidation

Platelet arachidonate peroxidation upon triggering with a supramaximal dose of collagen is measured by formation of malondialdehyde (MDA) as described by *Smith et al.* (25).
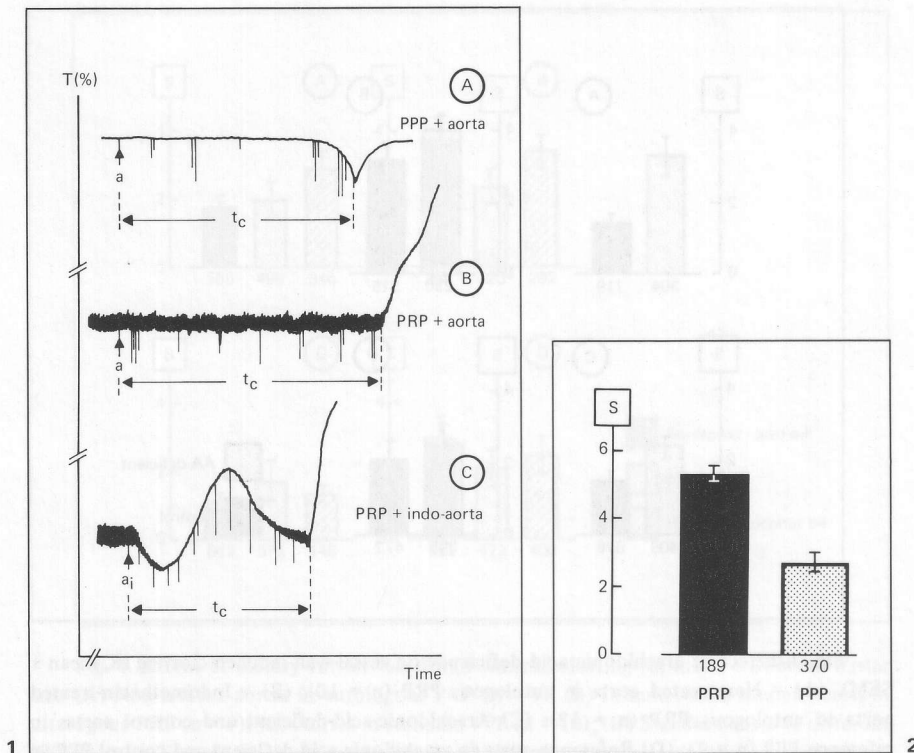
Fig. 1. Clotting-induced changes in light transmission (ΔT, %) in PPP (A) and PRP, preceded (C), or not (B), by platelet aggregation. 'Spikes' are caused by the tissue when passing the light beam; a = aorta; a_i = aorta, pretreated with indomethacin.

Fig. 2. Effect of the presence of platelets on vessel-wall-induced clotting. Figures below bars represent clotting times (s) calculated from mean S-values; n = 28; mean ± SEM.

Results and Discussion

Effect of Blood Platelets on Vessel-Wall-Induced Clotting

To establish whether the presence of platelets is a prerequisite for vessel-wall-induced coagulation, clotting times were determined upon incubation of aortic tissue in PRP and PPP, respectively. Clotting occurred in both media; however, in PRP it was significantly enhanced ($p_2 < 0.01$) as compared with PPP (fig. 2).
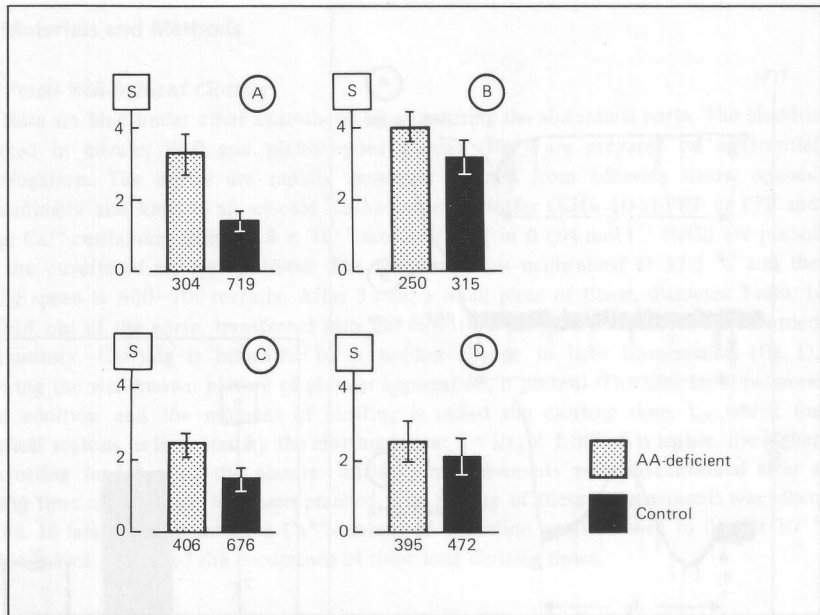
Fig. 3. Effect of arachidonic acid deficiency on vessel-wall-induced clotting (S, mean ± SEM). (A) = Non-treated aorta in autologous PRP (n = 10); (B) = Indomethacin-treated aorta in autologous PRP (n = 12); (C) Arachidonic-acid-deficient and control aortas in reference PPP (n = 7); (D) Reference aorta in arachidonic-acid-deficient and control PPP (n = 12). Figures below bars represent clotting times (s) calculated from mean S-values.

The aggregometer tracings of PRP did not show platelet shape change and aggregation, indicating that for this pro-coagulant activity, platelets not in direct contact with the vessel wall need not be activated. However, it remains possible that the adhered and thereby activated platelets are responsible for the clot-promoting effect observed.

Effect of Vascular Prostacyclin on Vessel-Wall-Induced Clotting

Although the injured vessel wall provides a thrombogenic surface, prosta-cyclin produced by the vascular tissue prevents activation of the non-adhering platelets. When prostacyclin formation is blocked, vessel-wall-induced clotting is always preceded by platelet shape change and aggregation. (fig. 1C). Two experiments were performed to investigate whether lowering of prostacyclin formation affects vessel-wall-induced clotting of PRP.
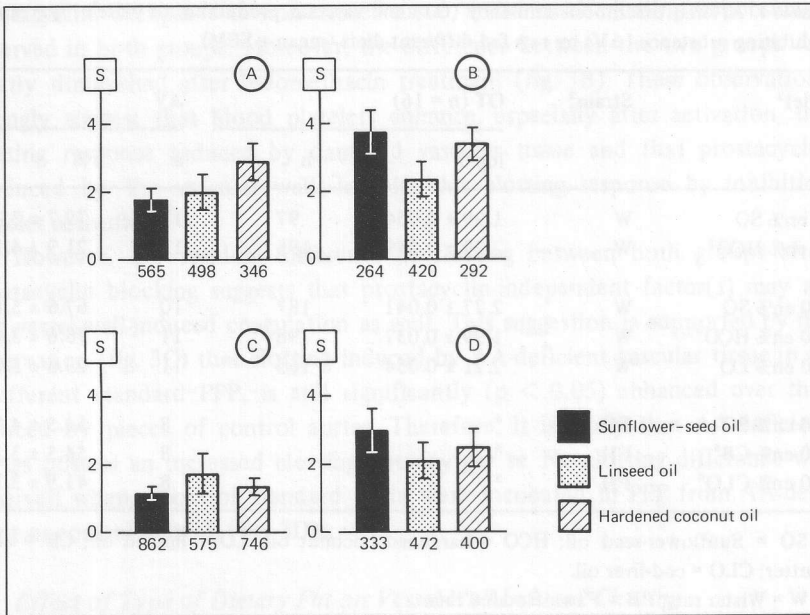
Fig. 4. Effect of dietary fat on vessel-wall-induced clotting (S, mean ± SEM) in Wistar rats. (A) Non-treated aortas in autologous PRP (n = 9); (B) Indomethacin-treated aortas in autologous PRP (n = 9); (C) Aortas in standard PPP (n = 10); (D) Standard aorta in different PPPs (n = 7). Figures below bars represent clotting times (s) calculated from mean S-values.

Effect of Drugs Inhibiting PGI_2 Synthesis

Vascular tissue was pre-incubated in KH buffer containing indomethacin (2 μg ml^{-1}), aspirin (100 μg ml^{-1}) or tranylcypromine (500 μg ml^{-1}). These treatments greatly diminished vascular prostacyclin production, caused platelet shape change and aggregation and enhanced vessel-wall-induced clotting in PRP (for indomethacin: see figs. 3 and 4). This indicates that prostacyclin may regulate vessel-wall-induced clotting possibly by inhibiting platelet activation. However, a direct effect of these drugs on vessel-wall-induced clotting cannot be excluded.

Effect of Arachidonic Acid Deficiency

Aortas of arachidonic-acid (AA)-deficient animals produce only small amounts of PGI_2 (7). So, if prostacyclin plays a regulating role in vessel-wall-induced clotting, this process might be expected to be enhanced in AA deficiency. Two groups of rats were used for the experiment: one group received a

Table I. Arterial thrombosis tendency (OT) and vascular production of platelet aggregation inhibiting substance (ΔV) by rats fed different diets (mean ± SEM)

Diet[1]	Strain[2]	OT (n = 16)		ΔV	
		log h	h	n	%
5 en% SO	W	1.99 ± 0.054	97	12	79.7 ± 3.77
5 en% HCO[3]	W	2.29 ± 0.049	195	12	21.9 ± 4.19
50 en% SO	W	2.27 ± 0.041	187	10	67.6 ± 5.82
40 en% HCO[4]	W	1.99 ± 0.037	98	11	76.6 ± 3.48
50 en% LO	W	2.21 ± 0.054	163	11	23.6 ± 5.64
50 en% SO	FH	[5]		8	51.5 ± 4.09[6]
50 en% CB[4]	FH	[5]		8	54.5 ± 3.16[6]
50 en% CLO[4]	FH	[5]		8	41.9 ± 3.77[6]

[1] SO = Sunflower-seed oil; HCO = hardened coconut oil; LO = linseed oil; CB = cocoa butter; CLO = cod-liver oil.
[2] W = Wistar rats; FH = Fawn Hooded rats.
[3] Arachidonic acid deficient.
[4] Dietary fat 45 en%; 5 en% SO added to prevent arachidonic acid deficiency.
[5] No reliable value, because circa 50% of the animals were lost due to bleeding problems or embolization of the arterial thrombus.
[6] Slightly different measuring procedure, leading to lower values. In fact, PGI_2-production in FH rats is significantly higher than in W rats.

diet containing 5 energy % (en%) sunflower-seed oil (control group) whereas the other group was fed a comparable diet with hardened coconut oil. This diet contains no linoleic acid, as a result of which it causes AA deficiency after a feeding period of about 3 months. Two series of clotting measurements were performed: one with a piece of normal, non-treated aorta and one with a piece of the same aorta, pretreated with indomethacin ($2 \mu g \, ml^{-1}$) for at least 1 h. Moreover, vascular prostacyclin production was measured, which confirmed the low PGI_2-producing potency of AA-deficient aortas (table I). In the first series of coagulation studies, clotting induced by AA-deficient aortas in autologous PRP, appeared to be significantly (p = 0.01) enhanced compared to that occurring with control material (fig. 3A). In the AA-deficient group, clotting was preceded by platelet shape change and aggregation. In the second series, indomethacin-treated aortas were used, thereby preventing any effect of vascular

prostacyclin. Compared to non-treated tissue, an increased clotting response was observed in both groups. Moreover, the difference between the two groups was greatly diminished after indomethacin treatment (fig. 3B). These observations strongly suggest that blood platelets enhance, especially after activation, the clotting response induced by damaged vascular tissue and that prostacyclin produced by the vascular wall, inhibits this clotting response by inhibiting platelet activation.

However, the residual difference in clotting between both groups after prostacyclin blocking suggests that prostacyclin-independent factor(s) may affect vessel-wall-induced coagulation as well. This suggestion is supported by the observation (fig. 3C) that clotting induced by AA-deficient vascular tissue in an indifferent standard PPP, is still significantly ($p < 0.05$) enhanced over that induced by pieces of control aortas. Therefore, it is likely that AA-deficient aortas possess an increased clotting potency *per se*. No clotting difference was observed when pieces of standard aorta were incubated in PPP from AA-deficient or control animals (fig. 3D).

Effect of Type of Dietary Fat on Vessel-Wall-Induced Clotting

To investigate whether the clotting response upon PRP-vessel wall interaction is influenced by the type of dietary fat, and if any dietary fat effect is mediated through the platelet release reaction, two sets of experiments were carried out: one in normal Wistar rats and one with release-deficient Fawn-Hooded rats.

Normal Wistar Rats. Three groups of rats were fed diets in which 50% of the energy was provided by either sunflower-seed oil (SO, mainly linoleic acid) linseed oil (LO, mainly linolenic acid) or hardened coconut oil (HCO, mainly saturated fatty acids). After 3 months, the arterial thrombosis tendency was determined in one half of the animals. In the other animals, the aortic wall-induced clotting in autologous PRP was measured, as were vascular prostacyclin production and platelet arachidonate peroxidation.

The results confirmed the differences in arterial thrombosis tendency and prostacyclin production (table I) found on earlier occasions (6). They also indicated clear differences in the mean clotting response of each group, the saturated fat group showing the highest and the linoleic acid-rich group, the lowest clotting tendency (fig. 4A). Due to wide individual variations, the differences did not reach the level of statistical significance. However, a striking relationship was observed between arterial thrombosis tendency and vessel-wall-
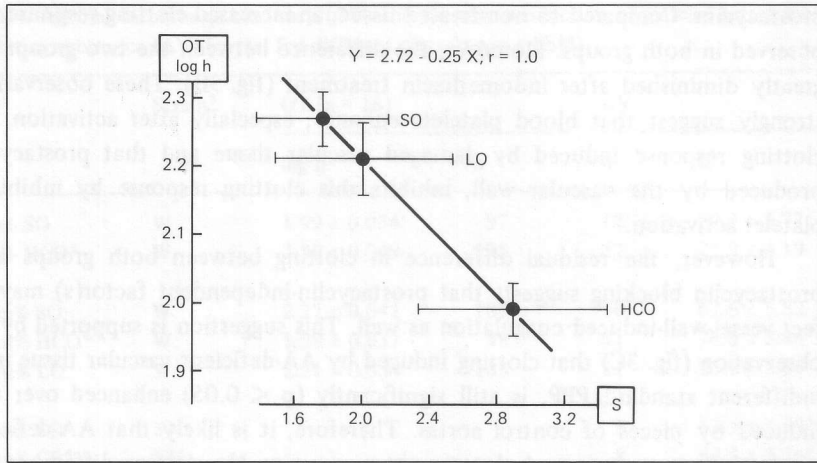
Fig. 5. Relationship between vessel-wall-induced clotting in autologous PRP (S ± SEM) and arterial thrombosis tendency (log OT ± SEM) measured in different groups of animals receiving 50 en% fat-containing diets. SO = sunflower-seed oil; LO = linseed oil; HCO = hardened coconut oil.

induced clotting (fig. 5), which indicates that this clotting response may contribute to arterial thrombus formation. When prostacyclin action is prevented by using indomethacin-treated aortas, coagulation in autologous PRP is enhanced in all three groups (fig. 4B). In the LO group, this enhancement is very small, leading to a significantly (p < 0.05) lower clotting tendency than in the other groups. Since no clotting differences were observed upon incubation of non-treated aortas in standard PPP (fig. 4C) or upon incubation of pieces of standard aorta in the different PPPs (fig. 4D), this must be due to a low 'activation grade' of the LO platelets induced by the vessel wall which can be either platelet- or aorta-bound. Most probably, the low 'activation grade' is caused by some platelet property, since arachidonate peroxidation upon supramaximal triggering with collagen appeared to be significantly lower than in the other groups (MDA formation in 10^{-9} mol per 10^9 platelets; LO: 0.78 ± 0.055; SO: 1.27 ± 0.118; HCO: 1.44 ± 0.080; n = 12, mean ± SEM).

Fawn-Hooded Rats. Fawn-Hooded (FH) rats have a hereditary defect of the platelet-release reaction: their dense granules, which, in normal animals, contain ATP, ADP, Ca^{++}, serotonin, pyrophosphate and antiplasmin (5), seem to be empty, leading to reduced platelet nucleotide and serotonin contents (21, 27).
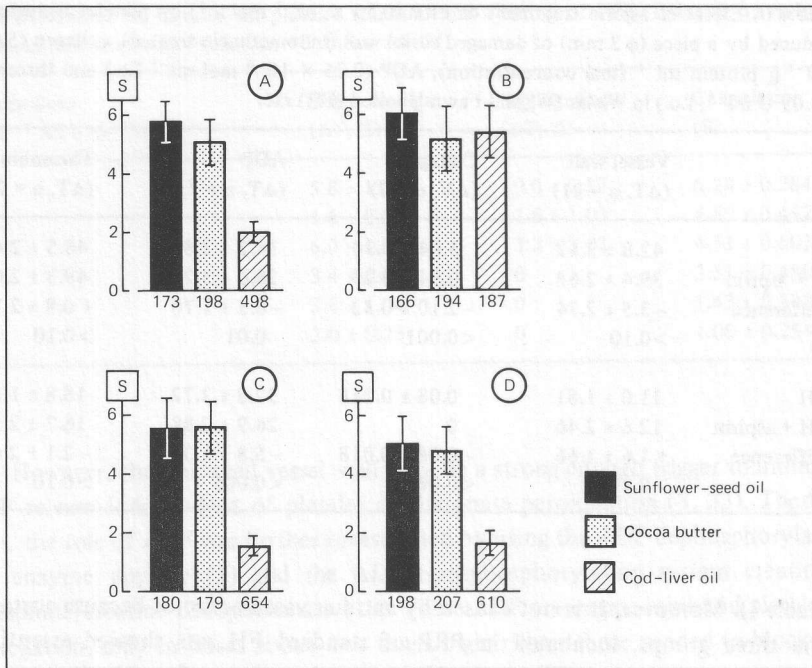
Fig. 6. Effect of type of dietary fat on vessel-wall-induced clotting (S ± SEM) in Fawn-Hooded rats (n = 8). (A) Aortas in autologous PRP; (B) Aortas in PRP of standard FH rats; (C) Standard FH aorta in different PRPs; (D) Standard FH aorta in different PPPs. Figures below bars represent clotting times (s) calculated from mean S-values.

This defect is thought to be the underlying mechanism of the bleeding tendency observed in these animals (27).

Three groups of FH rats were fed 50 en% fat-containing diets. SO was used as a linoleic acid-rich control, cocoa butter (CB) as a highly saturated fat and cod-liver oil (CLO) as a typical long-chain polyunsaturated oil. After 3 months' feeding, aortic-wall-induced clotting of PRP was measured, as was PGI$_2$ production by these aortas. In these 'release deficient' animals, vessel-wall-induced clotting of PRP is not enhanced by saturated fat feeding (fig. 6A) as was suggested to be the case in normal rats (fig. 4A). This discrepancy needs further investigation.

The very low clotting response in the CLO group (fig. 6A) cannot be due to vascular prostacyclin, because the total production of aggregation-inhibiting substances by the aortas of this group is lower than that in the other groups

Table II. Effect of aspirin treatment of PRP (0.55×10^{-3} mol l^{-1}) on platelet aggregation induced by a piece (ϕ 3 mm) of damaged vessel wall (indomethacin-treated), collagen (5.6×10^{-6} g protein ml^{-1} final concentration), ADP (0.25×10^{-6} mol ml^{-1} f.c.) and thrombin (0.09 U ml^{-1}, f.c.) in Wistar (W) and Fawn Hooded (FH) rats

	Vessel wall (ΔT, n = 21)	Collagen (ΔV, n = 7)	ADP (ΔT, n = 7)	Thrombin (ΔT, n = 7)
W	42.8 ± 3.12	3.04 ± 0.30	34.2 ± 2.81	48.5 ± 2.42
W + aspirin	39.4 ± 2.68	0.94 ± 0.29	28.0 ± 3.21	49.3 ± 2.68
Difference	-3.5 ± 2.74	-2.10 ± 0.32	-6.3 ± 1.70	$+0.8 \pm 2.72$
P_2	>0.10	<0.001	0.01	>0.10
FH	11.0 ± 1.81	0.08 ± 0.018	32.8 ± 2.72	18.8 ± 1.87
FH + aspirin	12.6 ± 2.46	0	26.9 ± 2.88	16.7 ± 2.54
Difference	$+1.6 \pm 1.66$	-0.08 ± 0.018	-5.8 ± 1.30	-2.1 ± 2.66
P_2	>0.10	<0.005	<0.005	>0.10

(table I). Moreover, it is not caused by another vascular factor because aortas of the three groups, incubated in PRP of standard FH rats showed essentially similar clotting potencies (fig. 6B). However, when pieces of standard aorta were incubated with different PRPs (fig. 6C) or PPPs (fig. 6D), clotting was always lowest in the CLO plasma, both other groups showing no difference at all. Therefore, it is very likely that the lower vessel-wall-induced clotting of PRP caused by CLO feeding can be ascribed to a plasma factor.

Vessel-Wall-Induced Platelet Reactions: Role of Thromboxane A_2 and ADP
Clotting of PRP, brought about by a piece of vascular tissue producing little or no prostacyclin, is preceded by platelet shape change and aggregation (fig. 1C). The time course of these platelet reactions very much resembles that of collagen-induced aggregation, which is mediated by Thromboxane A_2 (TxA$_2$, 24) produced by and ADP released from platelets that adhered to the collagen. This suggests that the vessel-wall-induced platelet reactions are mediated by the same processes. However, pre-incubation of PRP with aspirin (0.55×10^{-3} mol l^{-1}) for 1 h did not significantly reduce the vascular-tissue-induced platelet reactions, whereas ADP- and collagen-induced aggregation of PRP was significantly inhibited by this aspirin treatment (table II). Therefore, neither TxA$_2$ nor ADP seems to play a primary role in this type of platelet-vessel-wall interaction *in vitro*.

Table III. Effect of hirudin on platelet function and clotting of PRP, induced by indomethacin-treated vascular tissue (n = 5; mean ± SEM)

Hirudin dose ($\times 10^{-2}$ ATU ml^{-1} f.c.)	Shape change (ΔT, %)	Aggregation (ΔT, %)	Coagulation (S)
0	5.8 ± 1.39	19.0 ± 2.37	6.28 ± 0.284
3.125	4.6 ± 0.56	1.6 ± 1.03	4.89 ± 0.442
6.25	6.0 ± 1.55	3.2 ± 3.01	4.53 ± 0.603
12.5	3.4 ± 0.90	0	3.31 ± 0.480
25	3.8 ± 1.04	0	1.43 ± 0.333
50	2.0 ± 0.23	0	1.06 ± 0.254

However, the damaged vessel wall may be a strong enough trigger to induce ADP release independent of platelet arachidonate peroxidation (3, 33). Therefore, the role of ADP was further investigated by using the ADP dephosphorylating enzyme apyrase (1) and the ADP transphosphorylating system creatine phosphate/creatine phosphokinase (10). These ADP scavengers inhibited platelet aggregation, only in doses about two times higher than those needed to block a comparable degree of aggregation induced by added ADP.

Vessel-Wall-Induced Platelet Reactions: Role of Thrombin

Since the PRP-vessel-wall interaction triggers a clotting response, the preceding platelet reactions may be caused by thrombin. We tested this hypothesis by using hirudin, a potent and specific thrombin-inactivating polypeptide which does not modify ADP-induced aggregation. This substance very effectively blocks platelet aggregation when added to PRP in doses only slightly effective in lowering the clotting response. Hirudin appeared a less efficient inhibitor of platelet shape change (table III). As shown before (table II), the platelet response brought about by damaged vascular tissue is not inhibited by aspirin. The same aspirin treatment is equally ineffective in modifying thrombin-induced aggregation. Moreover, in PRP of FH rats, vessel-wall-induced platelet reactions are significantly less pronounced than in Wistar rats. The same holds for thrombin- but not for ADP- and collagen-induced aggregation (table II).

These striking similarities between vessel wall induced and thrombin-induced aggregation, together with the efficient inhibition by the anti-thrombin agent hirudin of aggregation induced by the damaged vascular wall, strongly suggests that this aggregation is mediated by thrombin, generated as a result of

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 adherent platelets and/or from non-adherent platelets, activated by the generated thrombin, cannot be excluded. In this respect, it seems relevant that the effective doses of these ADP-removing systems also inhibit thrombin-induced aggregation.

General Discussion

We demonstrated that injured vessel wall is able to clot plasma. It has not yet been established whether this process is of intrinsic or extrinsic origin. Since vessel wall preparations have a high tissue factor activity (for review, see ref. 17), extrinsic clotting seems the most likely process. However, preliminary experi-
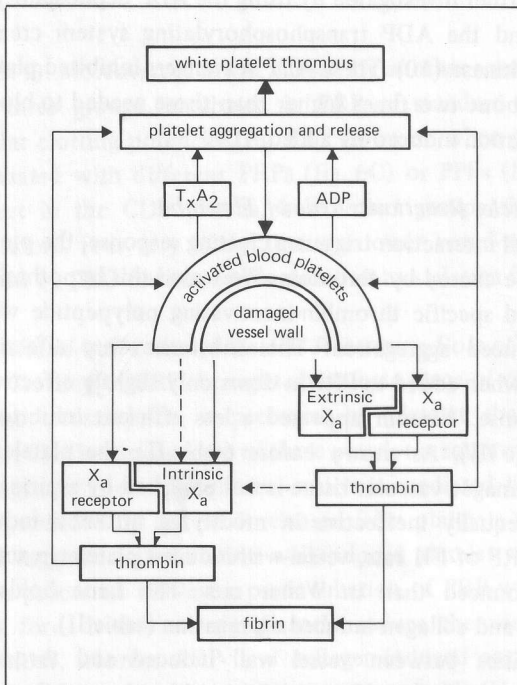

Fig. 7. Diagram to illustrate processes possibly involved in thrombus formation following vascular injury. For explanation: see text.

ments with factor-VII-deficient plasma were inconclusive, which might have been due to the perforce use of heterologous plasma in this type of experiment.

The intrinsic pathway of coagulation may also be involved in vessel-wall-induced clotting, since subendothelial collagen is able to activate factor XII (18, 32). Moreover, platelets which have been shown to enable the initiation (28, 29) and the stimulation (30, 31) of the intrinsic pathway, clearly stimulated vessel-wall-induced clotting (fig. 2). This effect did not require massive activation of the available platelets, as was demonstrated by the absence of platelet shape change and aggregation. This observation, together with recent findings (not shown) that aspirin treatment of PRP does not modify vessel-wall-induced clotting of this PRP, makes it rather unlikely that the enhancing effect of (activated) blood platelets on coagulation induced by vascular tissue is due to platelet factor 3 activity (4, 31).

If we consider our findings in the light of literature data, the following picture emerges as to the processes resulting from the interactions between damaged vessel wall and blood constituents (fig. 7). In the first reaction phase, the endothelial injury causes the adhesion of blood platelets to subendothelial structures, followed by the activation of the adhered platelets. Tissue factor activity of the injured blood vessel triggers the extrinsic pathway of coagulation, leading to the formation of activated factor X in close proximity to adhered blood platelets. These platelets, following activation, have disclosed specific receptors for activated factor X, as a result of which X_a binds to their surface (2, 12, 13). There is substantial evidence that activated factor V acts as this receptor (14, 20). Together with platelet membrane phospholipids and Ca^{++}, V_a accelerates the X_a-mediated proteolysis of prothrombin to thrombin at the platelet surface. Although, initially, the amount of thrombin generated in this way is only very small, the local concentration will be sufficient to uncover X_a receptors on fresh platelets. These have, in the meantime, concentrated around the vascular injury due to the aggregating activity of TxA_2 produced by, and ADP released from, the adhered platelets. This enables X_a to produce more thrombin. By activating factor VIII, thrombin may promote its own formation via the intrinsic clotting pathway (17), which is also initiated by the injured vessel wall. Via these positive feedback mechanisms, the local thrombin concentration is boosted. Since thrombin is a very potent platelet aggregating and releasing agent, it cooperates with TxA_2 and ADP in the production of a 'white platelet thrombus', the growth of which will be counteracted by prostacyclin generated in the vessel wall (15) from endogenous precursors (8, 34). The white thrombus has a fragile structure and embolizes very easily. However, thrombin

also mediates the formation of fibrin which consolidates the fragile platelet mass to a stable arterial thrombus of great pathological significance.

For a long time, the participation of coagulation in arterial thrombogenesis has been considered to be of secondary importance. However, it is now gradually becoming clear that white thrombus formation and blood clotting are two intimately linked processes equally important in arterial thrombogenesis. The effect of dietary fats on various platelet functions and on the presently described clotting response, induced by damaged vascular tissue, may be the basis of their influence on arterial thrombosis and atherosclerosis in animals and man.

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