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Interactions between stimulated platelets and endothelial cells *in vitro*

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Prostaglandins and hydroxy acids are synthesized mainly from the essential polyunsaturated fatty acid arachidonate, and these substances have been identified in almost all mammalian tissues. Prostaglandins, thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) are autocoids that appear to function in the regulation of vascular tone, cell secretion and contractile processes. So far, hydroxy acids have been found to function as chemotactic agents and in the formation of slow-reacting substances. Other actions of hydroxy acids will certainly be defined in future research. The endoperoxides PGG_2 and PGH_2 represent common precursors of all prostaglandin end-products. In studying the prostaglandin metabolism of a specific tissue, the total profile of endoperoxide transformation should be determined. In platelets the endoperoxides are transformed mainly into TXA_2, a potent vasoconstrictor and inducer of platelet aggregation. Endothelial cells convert endoperoxides to PGI_2, a vasodilator and inhibitor of platelet aggregation. In addition, endothelial cells can utilize endoperoxides from stimulated plates to form PGI_2. The concept that platelets and endothelial cells can share common precursors for the production of modulating substances may be applicable to other cell types.

Introduction

From the standpoint of both basic and clinical research, there is currently a great deal of interest in prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), the principal products of the cyclooxygenase pathways in endothelial cells and platelets, respectively (tables 1 and 2). PGI_2 induces strong vasodilatation and inhibits platelet aggregation by stimulating adenylate cyclase, which in turn elevates cyclic AMP and blocks platelet calcium mobilization. TXA_2 is a vasoconstrictor, inducing platelet aggregation via inhibition of adenylate cyclase and promotion of platelet calcium mobilization. Both PGI_2 and TXA_2 are derived enzymically from a common precursor, the prostaglandin endoperoxide PGH_2. The concept of reciprocal regulation of platelet cyclic AMP levels by PGI_2 and TXA_2 has interesting implications. As originally proposed by Bunting *et al.* (1976), a biochemical interaction between platelets and vessel walls may take place in which endoperoxides released in the immediate vicinity of stimulated platelets might be used by vessels to form PGI_2. This in turn would elevate platelet cyclic AMP. In this way a balance between the pro-aggregatory effect of TXA_2 and the anti-aggregatory activity of PGI_2 might be achieved (Marcus 1979).

In this paper we summarize recent research in our laboratory on interactions between stimulated platelets and cultured human endothelial cells. In addition, some aspects of arachidonic acid metabolism in platelets are discussed.

Table 1. Arachidonate metabolites from thrombin-stimulated endothelial cells

product	count/min	percentage
6-keto-$PGF_{1\alpha}$	116008	65.5
$PGF_{2\alpha}$	30484	17.2
PGE_2	6060	3.4
PGD_2	2119	1.2
hydroxy acids	6559	3.7
free 20:4	3220	1.8

Table 2. Arachidonate metabolites from thrombin-stimulated platelets

product	count/min	percentage
$PGF_{2\alpha}$	469	2.2
TXB_2	9268	43.1
PGE_2	467	2.2
PGD_2	607	2.8
hydroxy acids	9855	45.8
free 20:4	229	1.1

Arachidonic acid metabolism in human platelets

Arachidonic acid (20:4) is a polyunsaturated fatty acid, which mammals synthesize from linoleic acid (18:2), which cannot be synthesized by mammals and so must be obtained from the diet. Prolonged feeding of diets lacking in 18:2 and 18:3 to experimental animals results in a well characterized disorder known as essential fatty acid deficiency (for review see Marcus 1978).

Mechanical or biochemical stimulation of platelets initiates a series of biochemical events culminating in the transformation of arachidonic acid to TXA_2 and hydroxy fatty acids. Arachidonate composes about 30% of the fatty acids in platelet phospholipids (Marcus *et al.* 1969). Although arachidonic acid is present in all platelet subcellular compartments, i.e. membranes, granules and cytosol (Marcus *et al.* 1969; Broekman *et al.* 1976), the precise source of arachidonate for thromboxane synthesis is still not known. Arachidonic acid cannot be processed to TXA_2 or hydroxy fatty acids unless it is first rendered available in an unesterified form. Currently, two mechanisms for hydrolysis of platelet phospholipids to yield free 20:4 have received general acceptance. One involves the activity of a phospholipase C on platelet phosphatidylinositol, followed by hydrolysis of the resulting diglyceride by diglyceride lipase to yield unesterified arachidonic acid, which then becomes available to the cyclo-oxygenase and lipoxygenase enzymes (Bell & Majerus 1980; Broekman *et al.* 1980). The other mechanism involves a phospholipase A_2, which acts on arachidonate in the 2-position of one or more platelet phospholipids (Bills *et al.* 1977; Broekman *et al.* 1980). It is of interest that after platelet stimulation, far more 20:4 is available than is actually used by the cyclo-oxygenase and lipoxygenase enzymes. The insertion of molecular oxygen and subsequent rearrangement of arachidonate are catalysed by platelet cyclo-oxygenase. Arachidonic acid is the most important substrate for the cyclo-oxygenase, and Lands (1979) has proposed that hydroperoxide(s) is required for activation of cyclo-oxygenase. Some of the free arachidonate is bound to other proteins in the platelet cytoplasm, additional amounts are released into the surrounding

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medium, and some of the 20:4 becomes reacylated. These alternative pathways for the free arachidonate have not yet been investigated in human platelets.

Free arachidonate is also oxygenated by platelet lipoxygenase. The lipoxygenase is a cytoplasmic enzyme that catalyses formation of 12-hydroxy acids in the platelet. The rate of lipoxygenase catalysis is slower than that of cyclo-oxygenase. Thus, 12-hydroxy acids such as HETE appear in the milieu of stimulated platelets later than TXA_2 (Hamberg *et al.* 1974). HETE is a chemotactic molecule, and its late appearance may correlate with the delayed entry of leucocytes in haemostatic plugs and arterial thrombi.

Endoperoxide formation generates an interesting by-product, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT). This compound contains 17 carbon atoms, the other three being accounted for by malondialdehyde. As might have been anticipated, HHT and malondialdehyde do not form when platelets are pretreated with aspirin or indomethacin. Chemotactic activity is the only biological function to have been determined for HHT. Malondialdehyde was identified as thiobarbituric acid-positive material generated during thrombin-induced platelet aggregation (Hamberg *et al.* 1974), and is a highly reactive molecule that induces cross-linking of proteins and disturbances in enzyme function both *in vivo* and *in vitro*. Why this potentially harmful material is produced during platelet stimulation is not known.

Phospholipid metabolism in stimulated human platelets

Studies of endogenous phospholipid metabolism in stimulated platelets have recently been reported from our laboratory (Broekman *et al.* 1980). After platelets had been stimulated with thrombin or collagen, a phosphorus assay of the major and minor platelet phospholipids separated by means of two-dimensional thin-layer chromatography, was carried out. Endogenous platelet phosphatidylinositol (PI) rapidly decreased following thrombin addition. It was expected that the PI decrease would be accompanied by an increase in phosphatidic acid (PA). However, this correlation was not quantitative. One explanation for the discrepancy was that a transient intermediate in platelet PI metabolism, such as diglyceride formed by a PI-specific phospholipase C (Rittenhouse-Simmons 1979) was no longer available for PA formation as catalysed by diglyceride kinase. The transient diglyceride could have been hydrolysed by a diglyceride lipase, as recently described by Bell *et al.* (1979). The latter hydrolytic step would have yielded free arachidonate for subsequent transformation to products of cyclo-oxygenase and lipoxygenase activity. In addition, Broekman *et al.* (1980) noted accumulation of both choline and ethanolamine lysophosphoglycerides in stimulated platelets. Thus, after the addition of thrombin, lysophosphatidylethanolamine accumulated rapidly, reaching plateau levels within 15–20 s after stimulation. Analysis of the fatty acids and aldehydes of the lysophosphatidylethanolamine suggested the presence of platelet phospholipase A_2 activity with an apparent preference for diacylethanolamine phosphoglycerides. Broekman *et al.* also demonstrated that collagen stimulation of platelets was accompanied by changes in PI, PA and lysophospholipids, which occurred concomitantly with the aggregation response and consumption of oxygen indicative of prostaglandin endoperoxide formation. At present it is not unequivocally certain that arachidonate is released from platelet phospholipids by the pathway proposed by Bell *et al.* (1979), i.e. the action of a diglyceride lipase upon the product of phospholipase C hydrolysis of PI. The presence of a diglyceride lipase, combined with the well characterized loss in PI content due to phospholipase C (Rittenhouse-Simmons 1979) is

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suggestive, but more work remains to be done in this area. Our data on lysophosphoglycerides (Broekman *et al.* 1980) indicate that the Bell pathway is accompanied by classical phospholipase A_2 activity. On the other hand, our data showing incomplete recovery, as PA, of the loss in PI support the hypothesis of a diversion of an intermediate in the PI cycle, such as the action of diglyceride lipase on diglyceride produced by phospholipase C. However, other mechanisms are also possible, such as phospholipase(s) A_2 acting on PI and/or PA. These mechanisms are currently under study. Since the amounts of intermediate diglyceride are far less than the amounts of PI and PA, the diglyceride lipase would have to be far more active than possible phospholipase(s) A_2 acting on PI and/or PA. As yet no data have been presented supporting a turnover number that is sufficiently high. This should be viewed in the light of studies by Lapetina & Cuatrecasas (1979), who showed that PA may be the first lipid product formed after platelet stimulation. Further evidence supporting an important role for PA has been presented (Billah *et al.* 1979; Lapetina *et al.* 1980) and centres on the activity of diglyceride kinase. Obviously, competition between diglyceride kinase and diglyceride lipase could be crucial to the fate of diglyceride. Membrane perturbation with deoxycholate inhibits diglyceride kinase, leading to accumulation of diglyceride (Billah *et al.* 1979). Presumably diglyceride lipase activity was also blocked. Large concentrations of indomethacin (140 μM) inhibit diglyceride lipase more specifically, without inhibiting the kinase (Rittenhouse-Simmons 1980). To what extent these somewhat divergent sets of data are due to methodological and/or species differences is unknown. Our studies (Broekman *et al.* 1980) support phospholipase A_2 activity on PE and PC. In the absence of conclusive data we would suggest that both the Bell hypothesis and a role for PA are possible in human platelets. Whether PA may additionally function as a 'natural ionophore', as proposed by Gerrard *et al.* (1979), is an interesting question. Clearly more experimental data, both in intact platelets and in lysates, are necessary to establish a more definitive rank order for these interesting hypotheses.

Enzymic conversion of arachidonic acid and endoperoxide PGH_2 to prostacyclin by cultured human endothelial cells

Prostacyclin (PGI_2), the major prostaglandin produced by vascular tissues, is a strong vasodilator and the most powerful inhibitor of platelet aggregation yet described (for review see Marcus 1979). Formation of PGI_2 from the endoperoxide PGH_2 has been demonstrated in fresh arterial and venous tissues in several laboratories (for review see Moncada & Vane 1979).

In 1977, Weksler *et al.* demonstrated that cultured endothelial cells from human umbilical veins and bovine aorta generated an inhibitor of platelet aggregation. Microsomal fractions from these endothelial cells were then shown to synthesize PGI_2 after incubation with [^{3}H]-arachidonic acid (Weksler *et al.* 1977). Marcus *et al.* (1978) then carried out experiments in which exogenous radiolabelled PGH_2 or arachidonic acid was added to intact, unstimulated endothelial cell monolayers. Products of this reaction were studied in detail. The salient features of these studies are summarized here.

Incubation of endothelial cell monolayers with [1-^{14}C]*arachidonate*

When endothelial cell monolayers were incubated with radioactive arachidonate for 20 min, the major product in the supernatant was 6-keto-$PGF_{1\alpha}$, the chemically stable end-product of PGI_2 breakdown. Small quantities of $PGF_{2\alpha}$ and PGE_2 were also noted. The supernatant fluid

from these unstimulated monolayers also contained a large quantity of unconverted arachidonic acid. In contrast to the supernatants of the incubated cells, only traces of prostaglandins were detectable in extracts of the cells *per se*. Radioactivity associated with the cells was identified only in the phospholipid fraction and in the area on the thin-layer plates corresponding to unconverted arachidonic acid.

TABLE 3. QUANTITATIVE RADIO-T.L.C. ANALYSES OF SUPERNATANT PRODUCTS FORMED AFTER INCUBATION OF ENDOTHELIAL CELL MONOLAYERS WITH [1–^{14}C]PGH_2 AND [1-^{14}C]20:4

(After separation on t.l.c. plates, areas co-chromatographing with appropriate standards and representing radioactive peaks were scraped from the plates and counted. Numbers in the table represent the quantity of counts (± s.d.) in each peak as a percentage of the total counts recovered from the plate. The sum of the percentages does not equal 100 because there were scattered areas of insignificant radioactivity on the plate that did not correspond to known lipids.)

	cells			no cells	
product	20:4	20:4 + aspirin	PGH_2	20:4	PGH_2
6-keto-$PGF_{1\alpha}$	9.0 ± 3.0	0.7	14.6 ± 0.5	0.3	1.1†
$PGF_{2\alpha}$	3.4 ± 1.9	0.5	6.5 ± 2.0	0.2	2.0
PGE_2	4.8 ± 1.1	1.1	35.2 ± 2.3	0.3	46.3
PGD_2	—	—	18.2 ± 1.9	—	21.4
HHT	—	—	12.4 ± 5.0	—	12.9
Unconverted 20:4	65.6 ± 12.4	87.7	—	93.5	—

† Counts in the 6-keto-$PGF_{1\alpha}$ area of this plate did not represent a peak.

Incubation of endothelial cell monolayers with [1-^{14}C]PGH_2

In these experiments, endogenous prostaglandin synthesis was blocked by pretreatment of the cells with 100 μM acetylsalicylic acid. The radiolabelled PGH_2 was biosynthesized in our laboratory by the method of Gorman *et al.* (1977). Radiolabelled PGH_2 (2 μM) was added to the aspirin-treated endothelial cells for 5 min. Approximately 15% of the recoverable counts in the cell supernatants was accounted for by 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2 and HHT were also identified. Results obtained with the PGH_2 incubations were in contrast to those observed with arachidonate in that only 0.4% of the radioactivity recovered from the supernatants, plus the cells, were associated with the cells themselves.

Non-enzymic transformation of PGH_2 was studied by incubating radiolabelled PGH_2 in flasks that did not contain endothelial cells. No 6-keto-$PGF_{1\alpha}$ was identified in this system, but spontaneous formation of $PGF_{2\alpha}$, PGE_2, PGD_2 and HHT was observed. Results of the above experiments are summarized in table 3.

We proposed two hypotheses to explain the observed production of PGI_2 by resting endothelial cell monolayers (Marcus *et al.* 1978): (*a*) radiolabelled PGH_2 entered the cell, was converted to prostacyclin and other products, and was then released into the surrounding medium; (*b*) the radiolabelled PGH_2 was enzymatically and non-enzymatically transformed to PGI_2 and other prostaglandins at the cell surface. In any case, it was then of interest to extend these studies with the use of a natural source of PGH_2: the stimulated platelet. These studies have recently been completed and will be summarized in the next section. (Marcus *et al.* 1980).

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Use of platelet endoperoxides for PGI_2 production by endothelial cells

The experiments were carried out under conditions wherein stimuli were added to mixtures of platelets and endothelial cells. It was also necessary that the endothelial cells were unable to transform endogenous or exogenous arachidonic acid to prostaglandins or hydroxy acids. In control experiments, endothelial cells that had not been treated with aspirin were stimulated with thrombin in the presence of tritiated arachidonic acid (AA). Under these conditions, 66 % of the supernatant AA metabolites was 6-keto-$PGF_{1\alpha}$. Also present were $PGF_{2\alpha}$ (17 %), PGE_2 (3 %), PGD_2 (1 %), hydroxy acids (4 %), and unconverted arachidonic acid (2 %). When the endothelial cells were treated with aspirin (1 mM, 30 min) and then stimulated with thrombin in the presence of tritiated arachidonic acid, no radiolabelled 6-keto-$PGF_{1\alpha}$ was found as measured by radiochromatography and radioimmunoassay. These control experiments were carried out before, during and at the end of each experiment to ascertain that recovery from aspirin treatment did not occur.

An additional experiment to monitor endothelial cell recovery from aspirin treatment was performed as follows: radiolabelled platelets were pretreated with aspirin and then stimulated with thrombin in the presence of aspirin-inhibited endothelial cell suspensions. Thrombin stimulation resulted in the release of free arachidonate from the platelets, but this arachidonate was not used by the aspirin-treated endothelial cells, as shown by our failure to detect 6-keto-$PGF_{1\alpha}$.

PGI_2 *synthesis from platelet endoperoxides*

These experiments were carried out with the use of radiolabelled platelet suspensions ($2 \times 10^5/\mu l$) combined with 3000–6000/μl aspirin-treated endothelial cells, also in suspension. The mixtures were stimulated with ionophore A23187, thrombin or collagen in platelet aggregometry cuvettes. In this way, platelet aggregation responses and the formation of PGI_2 and TXA_2 were studied in the same sample.

Two additional controls were carried out, demonstrating that in the absence of an endoperoxide source, aspirin-treated endothelial cells did not form PGI_2. First, when endothelial cells were removed from the system, no 6-keto-$PGF_{1\alpha}$ was detected. Secondly, when unstimulated radiolabelled platelets were incubated with aspirin-treated endothelial cells, no PGI_2 production resulted.

When platelets and aspirin-treated endothelial cell suspensions were stimulated with ionophore, platelet aggregation was inhibited, and the radioactivity in thin-layer chromatograms of lipids derived from these incubation mixtures indicated formation of 6-keto-$PGF_{1\alpha}$. In addition, ionophore-treated platelets produced almost twice as much thromboxane B_2 (TXB_2) in the absence of endothelial cells than they did in their presence. The radiolabelling experiments were corroborated by the demonstration of significant quantities of 6-keto-$PGF_{1\alpha}$ by radioimmunoassay. Thus, a mixture of aspirin-treated endothelial cells (2×10^6) and platelets (10^8) produced 1.7 ng of 6-keto-$PGF_{1\alpha}$ after stimulation with ionophore. Despite the detection of 21 ng of TXB_2 in this mixture, no platelet aggregation occurred in the presence of endothelial cells.

In comparison with control samples, platelet aggregation in response to thrombin was always markedly reduced in the presence of aspirin-treated endothelial cell suspensions, and, con-

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comitantly, PGI_2 synthesis was demonstrable in these systems. Radioimmunoassay results indicated that 0.5 ng of 6-keto-$PGF_{1\alpha}$ and 7.6 ng of TXB_2 had formed after thrombin addition (table 4). As in the ionophore experiments, thrombin-stimulated platelets produced more TXB_2 (1.4-fold) in the absence of aspirin treated endothelial cells than in their presence.

In the presence of aspirin-treated endothelial cells, inhibition of the platelet aggregation response to collagen was comparable with that observed with thrombin. The quantities of 6-keto-$PGF_{1\alpha}$ and TXB_2 detected after collagen exposure were smaller than those observed with thrombin or ionophore. Thus, the quantitative profile of endothelial cell PGI_2 production in the presence of platelets was: ionophore > thrombin > collagen. The pattern of TXB_2 production by the platelets in these combined suspensions followed the same order.

TABLE 4. RADIOIMMUNOASSAY OF 6-KETO-$PGF_{1\alpha}$ AND TXB_2 PRODUCED BY SUSPENSIONS OF PLATELETS AND ASPIRIN-TREATED ENDOTHELIAL CELLS

product	ionophore	thrombin	collagen
6-keto-$PGF_{1\alpha}$/ng	1.7	0.5	0.1
TXB_2/ng	21.3	7.6	2.5

TABLE 5. PLATELET CONTRIBUTION TO PGI_2 FORMATION BY ENDOTHELIAL CELLS

components	6-keto-$PGF_{1\alpha}$/ng
endothelial cells + thrombin	7.7
endothelial cells + platelets + thrombin	19.2
aspirin-treated endothelial cells + platelets + thrombin	8.4

Contribution of platelet endoperoxides to PGI_2 *formation by endothelial cells*

Addition of thrombin to 3×10^6 endothelial cells in the absence of aspirin resulted in production of 8 ng of 6-keto-$PGF_{1\alpha}$. When 10^8 platelets were mixed with endothelial cells not treated with aspirin and stimulated with thrombin, 19.2 ng of 6-keto-$PGF_{1\alpha}$ was measured by radioimmunoassay. Thus the quantity of PGI_2 approximately doubled. Endothelial cells from the identical cultures were then treated with aspirin and added to platelets, the only source of endoperoxide. When this mixture was stimulated with thrombin, 8.4 ng of 6-keto-$PGF_{1\alpha}$ were detected, and this quantity was derived solely from platelet endoperoxides. This amount (8.4 ng) was similar to that produced by endothelial cells that had not been treated with aspirin. It was therefore concluded that in this system approximately half of the PGI_2 produced by platelet – endothelial cell mixtures originated from endoperoxides synthesized by platelets. These results are summarized in table 5.

Role of platelet concentration in the production of prostacyclin by aspirin-treated endothelial cells

A group of experiments were carried out wherein 1.45×10^6 endothelial cells (2900/μl) were combined with either 10^8 or 15.5×10^8 platelets (i.e. 2 or 31×10^5/μl), and each mixture was stimulated with ionophore, thrombin or collagen. The mixtures in which platelet concentrations were increased produced greater amounts of both 6-keto-$PGF_{1\alpha}$ and TXB_2. However, the increase in platelets resulted in the production of much more TXB_2 than 6-keto-$PGF_{1\alpha}$. The ratio of TXB_2 to 6-keto-$PGF_{1\alpha}$ was increased fourfold to sevenfold. The effects of increasing platelet concentrations in the setting of constant quantities of endothelial cells on the aggregation

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response were also evaluated. When 2×10^5 platelets/μl were combined with 3850 aspirin-treated endothelial cells/μl and the mixture was stimulated with thrombin (5 U/ml), platelet aggregation did not occur. On the other hand, when platelet concentrations were increased to 5×10^5/μl, the inhibitory effect of the aspirin-treated endothelial cells on platelet aggregation was lost and the responses were normal.

Comparison of studies involving platelets and endothelial cell monolayers with endothelial cell suspensions

When radiolabelled platelets were added to aspirin-treated endothelial cell monolayers and then stimulated with ionophore, less PGI_2 production occurred than when the experiment was carried out with platelets and endothelial cell suspensions (respectively, radioactive counts of 491 and 782/min). Comparable results were obtained when thrombin was the stimulus. When platelets and aspirin-treated endothelial cell monolayers were studied in the presence of thromboxane synthetase inhibitors such as imidazole or U54701, an increase in PGI_2 production occurred, as reported by other laboratories (Moncada *et al.* 1977; Needleman *et al.* 1979; Baenziger *et al.* 1979). Presumably these results were obtained as a consequence of an accumulation of platelet endoperoxides, thus facilitating PGI_2 synthesis by the aspirin-treated endothelial cells. These observations with thromboxane synthetase inhibitors suggested that endothelial cell synthesis of PGI_2 from platelet endoperoxides could indeed occur under appropriate experimental conditions.

The effects of imidazole and U54701 were readily apparent when platelets and aspirin-treated endothelial cell suspensions were used in place of the endothelial cell monolayers. Radioactive counts in the 6-keto-$PGF_{1\alpha}$ area of the thin-layer chromatograms increased from 782 to 12030/min in the presence of imidazole, and to 10847/min with U54701.

Possible significance of interactions between platelets and endothelial cells in vitro

There may be two mechanisms for PGI_2 synthesis by vascular tissues, and in particular human endothelial cells. The first involves synthesis of PGI_2 from endogenous precursors under conditions of perturbation of vascular surfaces. The second occurs from endoperoxides, which the endothelial cells can process when stimulated platelets are in close proximity. Specific conditions under which one or both of these mechanisms may be operative *in vivo* or even *in vitro* remain to be established.

In the studies reported, two independent methods for detection of 6-keto-$PGF_{1\alpha}$ were employed: radiolabelling and radioimmunoassay. The radiometric studies allowed us to trace the metabolism of platelet-derived endoperoxides by aspirin-treated endothelial cells. The radioimmunoassay experiments provided quantitative information on the total amount of 6-keto-$PGF_{1\alpha}$ generated by the endothelial cells. Furthermore, the radioimmunoassays allowed us to compare the quantity of 6-keto-$PGF_{1\alpha}$ synthesized endogenously by endothelial cells alone with that derived from platelet endoperoxides.

The use of [^{3}H]arachidonate of high specific activity in the radiolabelling experiments allowed us to study platelets in the concentration range of 2×10^5/μl and markedly increased the sensitivity of the thin-layer chromatographic detection system. The use of endothelial cell suspensions in preference to monolayers facilitated platelet – endothelial cell proximity by concentrating the components in a small volume and allowing adequate mixing. Under these conditions we were able to study aggregation responses and analyse the products generated in the same test sample.

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It was also of interest that stimulated platelets synthesized less TXB_2 when mixed with aspirin-treated endothelial cells than when present in the incubation system alone. One explanation is that some of the available platelet endoperoxides were diverted to PGI_2 synthesis when aspirin-treated endothelial cells were present with the platelets. In addition, it is plausible that the PGI_2 formed by the endothelial cells led immediately to inhibition of platelet aggregation and therefore to a decrease in additional thromboxane production.

Summary

1. We have demonstrated that aspirin-treated endothelial cells that were unable to synthesize prostacyclin from endogenous sources were capable of utilizing platelet endoperoxides for PGI_2 synthesis.

2. PGI_2 formation was demonstrable by radio-thin-layer chromatographic analysis and radioimmunoassay.

3. Inhibition of platelet aggregation took place in mixtures of aspirin-treated endothelial cells and platelet suspensions in which PGI_2 formation could be demonstrated.

4. Increasing the ratio of platelets to endothelial cells decreased the ratio of 6-keto-$PGF_{1\alpha}$ to thromboxane, and platelet aggregation occurred.

5. PGI_2 synthesis was more apparent in suspensions of platelets and aspirin-treated endothelial cells than it was when endothelial cell monolayers were studied.

6. PGI_2 synthesis was not attributable to recovery of endothelial cells from aspirin treatment.

7. Endothelial cells are capable of utilizing platelet endoperoxides for as much as one-half of their PGI_2 production.

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Discussion

I. F. Skidmore (*Glaxo Group Research, Ware, U.K.*).

1. In his graph of PI breakdown and PA accumulation Dr Marcus implied that the difference between the two might account for the arachidonic acid released. Is it possible to calculate how much arachidonic acid is released? Does the difference between PI breakdown and PA accumulation account for it, and if not, what other mechanisms are involved?

2. Some workers have claimed that lyso PC accumulates in activated platelets but Dr Marcus's data indicates that lyso PE is the major lysophosphatide found. How is this difference explained? Can it be attributed to differences in the time of sampling after activation?

3. Would Dr Marcus consider specific transacylation from PC (or PE) to PI as described by Irvine & Dawson (*Biochem. biophys. Res. Commun.* **91**, 1399–1405) as a reasonable alternative for the generation of lysophosphatides to a mechanism involving phospholipase A_2?

A. J. Marcus.

1. These are very interesting, indeed intriguing, questions. We inferred from our measurements of phosphate content that the difference between the loss in PI and the gain in PA might possibly contribute to the quantities of arachidonate hydrolysed from platelet phospholipids. Our calculations indicate that it probably can account for a maximum of two-thirds of the arachidonate liberated if we postulate that the quantities of diglyceride and other intermediates are negligible. Thus, other mechanisms, such as the phospholipase A_2 activity that we demonstrated on PE and PC, must also be contributing to the liberation of arachidonate. It is further possible to postulate pathways whereby PI is the sole source of the liberated arachidonate, with the loss of 20:4 from other phospholipids being channelled directly towards synthesis of PA and/or PI.

2. Technical differences, including sampling time and composition of the medium in which the platelets were suspended, are most likely responsible for this apparent discrepancy. Time-course studies, which are of course difficult to carry out, are critical in some of these determinations.

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3. This may indeed be a reasonable alternative but, it should be emphasized that Irvine & Dawson's work was carried out on rat liver microsomes. Nevertheless, this is a very interesting and, of course, energy-efficient process, which is currently under investigation in our laboratory.

G. Hornstra (*Department of Biochemistry, Rijksuniversiteit Limburg, The Netherlands*). Some time ago we published a study (Hornstra *et al.* 1979) in which we demonstrated that:

(*a*) the PGI_2 production of vascular tissue is not significantly different on incubation in either platelet-poor plasma (p.p.p.) or platelet-rich plasma (p.r.p.);

(*b*) indomethacin-treated tissue does not produce prostacyclin upon short-term incubation in p.p.p. or p.r.p., whereas it does so on incubation with PGH_2;

(*c*) vascular tissue pretreated with indomethacin is unable to produce prostacyclin even if it is incubated in a suspension of collagen-activated endoperoxide-producing blood platelets;

(*d*) PGI_2 formation of vascular tissue is not different upon incubation in collagen-activated p.r.p. of normal or arachidonic acid-deficient rats producing very different amounts of endoperoxides.

We also demonstrated that the restoration of prostacyclin production occurring upon long-term incubation of indomethacin-treated vessel walls as observed by Bunting *et al.* (1976) is due to the removal of indomethacin from the cyclo-oxygenase enzyme system, which is consequently reactivated.

Recently we have shown that the presence of blood platelets does not significantly accelerate the course of this restoration.

It might be true that our negative results are due to the fact that the ratio between the number of platelets and endothelial cells was too high to allow platelet-derived endoperoxides to reach the vascular tissue. However, it should be realized that such a high ratio will also exist at the site of mural thrombus formation. Nevertheless, Dr Marcus has very convincingly shown that under favourable conditions endoperoxides can indeed escape platelets and serve as a substrate for vascular prostacyclin formation. The question that remains to be answered is: does this also happen under conditions less artificial than used so far?

Reference

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