CULTURED HUMAN SKIN FIBROBLASTS AND ARTERIAL CELLS PRODUCE A LABILE PLATELET-INHIBITORY PROSTAGLANDIN

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Summary

Human skin fibroblasts and cells cultured from human arterial smooth muscle produce a platelet-inhibitory prostaglandin in response to mechanical trauma. This prostaglandin is synthesized from an endogenous precursor rather than exogenous cyclic endoperoxides; it differs from PGE_1 and PGD_2 and resembles PGI_2 (prostacyclin) in its stability properties, being stable at pH $\geq$ 8.5 and labile at pH 7.4 and below. The prostaglandin synthesis pathway in these cultured cells is less sensitive to inhibition by aspirin than that in human platelets.

Introduction

The prostaglandin biosynthetic pathway in human platelets appears to play an augmenting role in hemostasis through the production of thromboxane A_2 and its cyclic endoperoxide precursors PGG_2 and PGH_2; all three are short-lived intermediates which cause vasoconstriction and platelet aggregation (1,2). Platelet prostaglandin synthesis is irreversibly blocked by aspirin via a covalent acetylation of the pathway's first enzyme, arachidonic acid cyclooxygenase (EC1.14.99.1) (3). Aspirin is currently being tested as a potentially useful drug in preventing myocardial infarction (4).

Certain other prostaglandin metabolites are known to inhibit platelet aggregation, including PGE_1, PGD_2, and PGI_2 or prostacyclin, a recently described labile prostaglandin produced in vascular tissue (5-8). Vane and co-workers have proposed that this latter inhibitory prostaglandin is synthesized in endothelial cells lining vessel walls, utilizing cyclic endoperoxide precursors elaborated by platelets and thereby preventing platelet aggregation in an intact vascular system (7,8). According to this view, aspirin therapy may be

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detrimental rather than helpful if it prevents the synthesis of the protective PGI_2 by inhibiting cyclic endoperoxide production from platelets.

We report here that cultures of human skin fibroblasts and cells derived from the smooth muscle of human aortic media produce a platelet-inhibitory prostaglandin in response to mechanical trauma. This inhibitory prostaglandin resembles PGI_2 in its stability properties and is synthesized from endogenous precursors rather than from platelet-derived cyclic endoperoxides. Production of the inhibitory prostaglandin by these tissue culture cells is inhibited by aspirin, but the inhibition requires much higher aspirin concentrations than those which inactivate the platelet enzyme. Thus, *in vitro* levels of aspirin can be found where platelet cyclo-oxygenase is completely inactivated and yet the cultured cells' capacity to synthesize the inhibitory prostaglandin remains intact.

Materials and Methods

Cell Culture: Diploid human skin fibroblasts RoBel (American Type Culture Collection) and GM-288 (Institute for Medical Research, Camden, N.J.) were grown in 100 mm dishes (Falcon) in Dulbecco's and McCoy's 5a media, respectively, with 20% fetal calf serum (Gibco). Arterial cells designated 11S07 were derived from explants of human abdominal aorta according to the method of Ross (9); they were grown in Dulbecco's medium modified for smooth muscle cells and show morphological and culture growth properties reported for the latter cells (9,10).

Measurement of Inhibitory Prostaglandin Production in Cultured Cells: Cells were assayed for prostaglandin production at confluence 7-11 days after plating and 1-2 days after a medium change. Cell layers were washed 3 times in serum-free culture medium or PRB[1] and scraped from the dishes with a rubber policeman in pH 8.6 PRB. After 5 minutes at 22°C the mixture was centrifuged 1 minute at 12,000 x g (Brinkmann 3200) and aliquots of supernatant were added to [^{14}C]serotonin-loaded platelets (5 x 10^7/ml) and incubated 2-10 minutes at 22°C prior to addition of purified human thrombin. After 2 minutes [^{14}C]serotonin retained in the platelets was measured by filtration as described previously (11,12). In studies of the lability of PGD_2, PGE_1, and the inhibitory prostaglandin as a function of pH, the prostaglandins were initially in pH 8.6 PRB which was titrated with 0.5 N acetic acid to pH 7.4 or pH 4. In the latter case neutrality was restored with 1 M Tris base before adding the solution to platelets.

Aspirin Sensitivity of Cyclo-Oxygenase in Platelets and Cultured Cells: Confluent cell cultures (100 mm) were washed twice with serum-free medium and incubated with aspirin (serial dilutions of 0.1 M ethanol solution in serum-

[1] PRB, 0.14 M NaCl-0.0055 M glucose - 0.015 M TrisCl at either pH 7.4 (11) or 8.6 as indicated; MDA, malonaldehyde.

free medium) for 20 minutes at 37°C in 5% CO_2-air, washed twice, scraped in pH 8.6 PRB, and centrifuged after 5 minutes. Aliquots of supernatant were assayed for inhibition of platelet serotonin release. Platelets in pH 7.4 PRB (11) (7.5×10^8/0.5 ml) were treated for 20 minutes at 37°C with aspirin (2.5 µl of ethanolic solution). Arachidonic acid (2.5 µl of 5 mM ethanol solution, final 25 µM) was added and MDA production at 2 minutes was measured as described previously (3). Aspirin itself has no direct effect on the thrombin dose response curve for platelet [^{14}C]serotonin release.

Results

Production of a Platelet Inhibitor in Cultured Arterial Cells: In over 30 experiments using a previously described (11,12) assay based on the thrombin-induced release of [^{14}C]serotonin from human platelets, we have detected an activity in cultured human arterial cells which inhibits this release. Figure 1a indicates the dose response curve for thrombin-induced serotonin release in control platelets. When platelets were incubated with increasing amounts of pH 8.6 PRB supernatant derived from cells mechanically dislodged from the culture dish, the thrombin dose response curve was shifted so that a higher thrombin concentration was required to effect half-maximal [^{14}C]serotonin release. In Figure 1b the percent shift in the thrombin dose response curve of Figure 1a has been plotted against the amount of supernatant added; the shift was linear over a range of 300-2500% in this experiment, and a similar linear relationship was found for the supernatant from scraped human skin fibroblasts in two other experiments. The percent shift produced by a given number of cultured cells ranged from 300-400% up to 1100-2500% in different experiments; however, within each individual experiment using a set of identical cultures the shift was very reproducible.

Characterization of the Inhibitor as a Labile Prostaglandin: Production of this dialyzable inhibitor was complete within 5 minutes after scraping the cells, and its synthesis was totally inhibited by treatment of the cell cultures with aspirin prior to scraping, indicating that it is a prostaglandin metabolite. No basal inhibitory activity was detected in undisturbed cell layers. A similar pattern of synthesis in a short-lived burst induced by mechanical trauma has been reported for prostaglandin synthesis in cultured

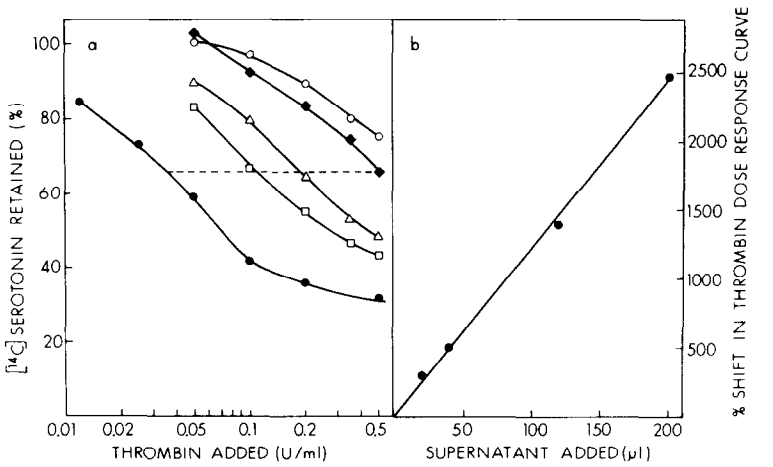

Figure 1. Production by 11S07 human arterial cells of a prostaglandin which inhibits thrombin-induced release of [^{14}C]serotonin from human platelets. Cells were scraped from culture dishes and the supernatant assayed as in Methods. a) Control platelets: ●—● ; platelets + supernatant: □—□ 20 µl, △—△ 40 µl, ◆—◆ 120 µl, ○—○ 200 µl. Dashed line indicates half-maximal serotonin release. 4) Percent shift in the dose response curve is derived from a); 200 µl supernatant contains the amount of inhibitor produced by approximately 5 x 10^5 cells.

mouse fibroblasts (13). The inhibitory prostaglandin from human arterial cells and skin fibroblasts was stable for over 40 minutes at 22°C and pH 8.6. However, at pH 7.4 the activity declined 87% in the same length of time and disappeared within 10 minutes of incubation at 37°C. When incubated for 30 minutes at 22°C and pH 4.0 the prostaglandin lost 87-91% of the activity seen at pH 8.6. This is in striking contrast to PGD_2 and PGE_1; at concentrations of 4.4 ng/ml and 20 ng/ml, respectively, these prostaglandins gave the same relative shift as the prostaglandin produced by 5 x 10^5 scraped cells and their activities were stable at all the temperatures and pH values listed above. Therefore, the inhibitory prostaglandin from cultured human cells differs from PGD_2 and PGE_1 and resembles PGI_2, which is stable only at pH $\geq$ 8.5 (14); however, no unequivocal identification has yet been made.

Aspirin Sensitivity of Prostaglandin Synthesis in Platelets and Cultured Cells: The relative sensitivities of platelet, fibroblast, and arterial cell cyclo-oxygenase to inactivation by aspirin were estimated by treating all three

TABLE II

The Effect of Triglyceride Bound and Unbound

Arginine Rich Apoprotein on Rat Lipoprotein Lipase

Experiment	Lipase Activity[a] nmol/hr/ml	
	Uncentrifuged Substrate	Centrifuged Substrate
Control (No ARP[b])	0.31	0.34
ARP 33 (100 µg/ml)	0.09	0.59
ARP 426 (100 µg/ml)	0.06	0.48
ARP 518 (100 µg/ml)	0.09	0.54
ARP 21 (100 µg/ml)	0.07	0.61

[a] The activity figures represent the means of duplicate determinations of lipoprotein lipase assayed as described in the text.

[b] ARP = arginine rich apoprotein.

(Figure 1). The bulk of the apoprotein (>80%) remained in the infranate unassociated with glyceride radioactivity. These triglyceride emulsions which had been exposed to the arginine rich apoprotein appeared to take up the apoprotein as does the native lymph chylomicron when it enters the plasma (6). When this centrifuged substrate, containing arginine rich protein, was incubated with the enzyme a reproducible increment in enzyme activity was observed (Table II). The centrifuged substrate demonstrated almost twice the activity of the control centrifuged substrate and was almost eight times more active than the uncentrifuged substrate which had the bulk of the arginine rich apoprotein in the substrate unbound form.

DISCUSSION

A number of observations have suggested that the arginine rich apoprotein has a function in the plasma transport of triglycerides. It has been noted

higher aspirin level (50-100 μM) fibroblasts and arterial cells still produced 40-55% of the inhibitory prostaglandin. A similar decreased aspirin sensitivity relative to that of the platelet enzyme has been reported for sheep seminal vesicle cyclo-oxygenase (16) and PGE synthesis in dog cardiac muscle (17).

Discussion

The recent discovery of PGI_2 has suggested a possible system of checks and balances between prostaglandins formed in platelets which promote platelet aggregation (thromboxane A_2) and those formed in vascular tissues which inhibit it (PGI_2) and thus may protect the vessel wall from thrombotic events (18). The relative importance of PGI_2 synthesis from endogenous arachidonic acid present in vascular tissues is unknown, and current models have stressed the role of platelets as a source of cyclic endoperoxides for PGI_2 synthesis by the vessel wall (7,18). However, the inhibitory prostaglandin produced by arterial media cells and skin fibroblasts is clearly a product of an endogenous precursor, since it is found in the supernatant from scraped cells which have had no contact with platelet-derived cyclic endoperoxides. Sufficient inhibitory prostaglandin can be generated in 11S07 arterial cells via endogenous synthesis to increase the required thrombin dose for half-maximal platelet serotonin release by 25-fold (2500%, Fig. 1b).

It should be noted that 11S07 arterial cells are derived from the medial region of the artery underlying the endothelium, and would therefore be the cells exposed to adhering platelets in areas of endothelial cell loss due to injury. Although endothelial cells have been proposed as the primary source of PGI_2 in blood vessels, little direct supporting evidence is available (8, 19). It would be interesting to compare the production of PGI_2 by human arteria endothelial cells and smooth muscle cells and to determine the relative aspirin inhibition of PGI_2 production in both cells. A suitable tissue culture system for human arterial endothelial cells has not yet been developed for this comparison. Our present finding of a platelet-inhibitory prostaglandin in cultured cells from the arterial media as well as in skin fibroblasts suggests

that the synthesis of platelet-inhibitory prostaglandins could be a general mechanism for regulating the interaction between platelets and a variety of tissues which they contact at sites of injury.

Prostaglandin synthesis in both arterial cells and skin fibroblasts is clearly less sensitive to aspirin than is platelet cyclo-oxyenase activity *in vitro*. *In vivo* studies measuring platelet cyclo-oxygenase inactivation after oral doses of aspirin indicate that 20 mg of aspirin per day, a dose well below the anti-inflammatory range, is sufficient to inactivate platelet cyclo-oxygenase by 61%; complete inactivation was seen at 320 mg per day (16). The unique sensitivity of platelet cyclo-oxygenase to aspirin inhibition suggests that an *in vivo* level of aspirin can be determined which will effectively inhibit platelet cyclo-oxygenase without compromising the same enzyme in other tissues which may be synthesizing protective prostaglandins. That some human tissues are very resistant to inhibition of PG synthesis by aspirin is known from studies where large doses of aspirin (up to 7 gm/day) fail to completely eradicate prostaglandin production in man (20,21). Whether an aspirin dose low enough to selectively inhibit platelet prostaglandin synthesis can actually prevent thrombosis in man can only be determined by clinical trials.

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