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Restoration of prostacyclin synthase in vascular smooth muscle cells after aspirin treatment: regulation by epidermal growth factor

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Abstract Prostacyclin is a potent vasodilator and inhibitor of platelet aggregation and plays an important role in maintenance of vascular homeostasis. Aspirin irreversibly inactivates prostacyclin synthetase by acetylating the enzyme. Recovery of the enzyme following inactivation by aspirin was studied in rat aorta smooth muscle cells in tissue culture. Confluent cultures superfused with [^{14}C]arachidonic acid, synthesized prostacyclin (PGI_2) together with prostaglandins E_2, D_2, and $F_{2\alpha}$. Brief treatment with physiological levels of aspirin (0.2 mM) completely inactivated prostacyclin synthesis. Following aspirin removal and addition of fresh growth medium, PGI_2 synthesis recovered rapidly with a $T_{1/2}$ of only 30–40 min, compared to a doubling time of 24–30 hr for the cells. Recovery of PGE_2, PGD_2, and $PGF_{2\alpha}$ synthesis paralleled that of PGI_2, confirming that cyclooxygenase rather than endoperoxide-prostacyclin isomerase was the labile component. Recovery of PGI_2 synthesis after aspirin was blocked by cycloheximide but not by actinomycin D. Recovery of aspirin-inactivated cells required a non-dialyzable component present in serum. All samples tested, including fetal bovine, new-born calf, human, and guinea pig, showed the activity. Fresh serum also induced a cycloheximide-sensitive 2- to 3-fold increase in cyclooxygenase levels in resting confluent cells within 1 to 2 hr. Serum factor was also required to restore PG synthesis after aspirin-inactivation in other cells, including 3T3 mouse fibroblasts, SV40-3T3 and K-Balb 3T3 transformed mouse fibroblasts, NRK rat kidney cells, and REF-9 rat embryonic fibroblasts. The activity was thermolabile, and was completely removed from the medium by growing cells. The material was recovered in protein following delipidation of serum, in the impermeate following filtration through molecular filters, and also in the infranatant after removal of lipoproteins by ultracentrifugation. Activity was not duplicated by α-tocopherol, estradiol, or insulin, or by platelet (PDGF) and fibroblast (FGF) growth factors. However, nanogram quantities of purified mouse epidermal growth factor (EGF) effectively replaced the biological activity of 10% serum. Maximum stimulation occurred at an EGF concentration, in serum-free chemically defined medium, of approximately 40 ng/ml. These studies indicate that synthesis of cyclooxygenase in many cells requires EGF. In aspirin-inactivated cells, EGF induces rapid restoration of the enzyme by a cycloheximide-sensitive process that does not require synthesis of new mRNA. Refractoriness to PG-releasing stimuli develops due to inactivation of cyclooxygenase; EGF may also be involved in regeneration of prostaglandin synthetase in vivo. —**Bailey, J. M., B. Muza, T. Hla, and K. Salata.** Restoration of prostacyclin synthase in vascular smooth muscle cells after aspirin treatment: regulation by epidermal growth factor. *J. Lipid Res.* 1985. **26:** 54–61.

Supplementary key words arachidonic acid • cyclooxygenase

Arachidonic acid is the precursor of a diverse range of potent biological effector molecules. These are of two main types. The first type, which includes the prostaglandins, thromboxanes, and prostacyclins, is synthesized via cyclooxygenase enzymes through a common endoperoxide precursor (1). The second group, including the mono HETEs and leukotrienes, is synthesized by lipoxygenases, the primary precursor being a hydroperoxy fatty acid (2).

Many of the nonsteroid anti-inflammatory drugs function by inhibiting the cyclooxygenase enzyme (3), thus preventing synthesis of the prostaglandin family of compounds. Aspirin, the most widely used of these agents, irreversibly inactivates the enzyme by acetylating a serine residue in the active site (4). Thromboxane A_2 synthesized by platelets is a potent aggregating agent (5). Aspirin, by blocking the synthesis of thromboxane, is a potent inhibitor of platelet aggregation (6).

Platelets are believed to be involved at several stages in the development of atherosclerosis both by releasing proliferative substances for smooth muscle cells and by initiating thrombus formation (7). In addition, the atherogenic process has many features of a tissue inflammatory reaction (8). These observations have led to the initiation of several clinical trials to test various

Abbreviations: PG, prostaglandin; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; HNCTC-135, NCTC-135 growth medium; 15-HETE, 15-hydroxyeicosatetraenoic acid; RB cells, rat aorta smooth muscle cells; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

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anti-inflammatory drugs, including aspirin, in individuals who had already suffered one heart attack (9, 10).

While these trials were in progress Moncada et al. (11) reported the identification of prostacyclin (PGI_2) as a potent inhibitor of platelet aggregation. This compound, which is synthesized in blood vessels via the cyclooxygenase pathway, is believed to be responsible for the well-recognized property of vascular tissue to resist platelet adhesion and aggregation. It is apparent that inactivation of vascular prostacyclin synthetase by aspirin could oppose the beneficial effects of aspirin on the platelets per se. It has been suggested that these effects may account for the equivocal results observed in those clinical trials using aspirin (12). Platelets, since they lack the necessary protein-synthesizing ability, are essentially inactivated for the remainder of their circulating lifetime (about 10 days) following a single exposure to aspirin. Vascular cells, however, since they can synthesize new protein, can presumably recover after aspirin treatment.

Prostacyclin synthesis has been demonstrated in cultured vascular endothelial cells (13–16) and smooth muscle cells (17–19), and shown to be inhibited by aspirin (20) and other nonsteroid anti-inflammatory drugs (21).

The present study was undertaken to characterize the synthesis of prostacyclin and related metabolites by cultured vascular smooth muscle cells before and after inactivation by aspirin, to investigate, in particular, the recovery of prostacyclin synthesis following aspirin removal, and to study the mechanism of recovery with particular reference to its requirement for macromolecular serum factors.

MATERIALS AND METHODS

Cell culture procedures

Confluent cultures of rat aorta smooth muscle cells were isolated from the aortas of Wistar rats by sequential elution of the cannulated vessel with collagenase and trypsin (17). Cells were maintained in 25-sq cm or 75-sq cm flasks (Costar); for assay of prostacyclin synthesis following aspirin treatment, cells were plated out in replicate cultures in 10-sq cm or 25-sq cm culture plates (Costar). Cells were grown in NCTC-135 (Flow Laboratories) medium buffered with HEPES (Fisher) (hereinafter referred to as HNCTC-135) and supplemented with 10% fetal bovine serum (M. A. Bioproducts). Antibiotics (Gibco) gentamycin (50 μg/ml), penicillin (50 units/ml), and streptomycin (50 μg/ml) were added to all cultures. Cells were harvested for subculturing using 0.25% trypsin (Gibco), in calcium-free (CMF) Hanks medium plus EDTA (0.54 mM) and incubated at 37°C for 2–4 min to release the cells. Cell populations were determined using a Coulter Counter and cell protein content was measured by the procedure of Lowry et al. (22). Serum-containing growth medium was then added and the resulting suspension was aliquotted into culture flasks or wells at a subculturing ratio of 1:4. Cultures became confluent within 3–4 days at which time they were used for assay of prostacyclin formation as described below.

Inactivation of cells by aspirin was carried out by replacing the growth medium with HNCTC-135 medium containing a previously prepared solution of aspirin (VSP) in a final concentration of 200 μM. The cells were incubated for 30 min at 37°C, following which the aspirin-containing medium was removed and the cells were washed twice before the test medium was added. Cultures were harvested at intervals and tested for recovery of ability to synthesize prostacyclin as described below. Test compounds cycloheximide, actinomycin D, EGF, or FGF (Collaborative Research Inc., Lexington, MA) and apolipoproteins were dissolved in HNCTC-135 before addition to the medium. α-Tocopherol and serum lipids were dissolved in ethanol, 1–5 μl per culture, and appropriate additions of alcohol were made to control cultures.

Incubation of cells with [^{14}C] arachidonic acid and product extraction

Medium was removed from confluent cultures and the cells were washed twice with HNCTC-135 (pH 7.4) at 37°C using 2 × 1 ml portions for 25 cm^2 flasks and 2 × 0.5 ml for well cultures. [1-^{14}C]Arachidonic acid (0.75 μCi, 4 μg per ml) was added as follows: 1 ml per 25-sq cm flask, 0.5 ml per 10-sq cm well, and 0.25 ml per 2-sq cm well. All cultures were incubated at 37°C for 5 min, the medium was collected and added to tubes containing 5 μg each of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2, and 15-HETE as carriers. Samples were acidified to pH 3 with 1 N HCl and extracted three times with two volumes of ethyl acetate or once with six volumes of chloroform–methanol 2:1. Ethyl acetate layers were collected and backwashed with one volume of distilled water. When extracting media containing serum, samples were extracted with six volumes of chloroform–methanol; the methanol-aqueous layer was extracted twice more with chloroform and the organic layers were separated and stored at −20°C until analyzed.

Thin-layer chromatography–radioautography procedures

Silica gel G TLC plates (Analtech) were used to separate prostaglandins and hydroxy fatty acids, using solvent system Iw (17), which consisted of the organic layer of a mixture of ethyl acetate–isooctane–water–acetic acid 11:5:10:2. After sample application, plates

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were equilibrated in water vapor for 30 min before development. A separate lane of TLC standards was included with each plate and the compounds in this lane only were visualized by spraying with 10% phosphomolybdic acid in methanol followed by heating at 110°C for 5 min. Radioactive compounds were analyzed by three different procedures. The plates were first scanned using a Vanguard radioactivity scanner (Model 930 Autoscanner, scanner gas 1.3% butane in helium) with an efficiency for ^{14}C of approximately 20%. The plates were then wrapped in plastic wrap (Union Carbide) and placed on 8" × 10" sheets of XAR X-OMat X-ray film (Kodak) in the dark for 5–7 days. The exposed film was developed for 5 min in Kodak X-ray developer at 25°C and fixed in Kodak Rapid Fix for 3 min. After separation by TLC and visualization by radioautography, the radioactive bands were tentatively identified by comparison with the authentic standards and scraped from the plates into 4 ml of Aquasol (New England Nuclear) for quantitative determination by liquid scintillation counting. All data were converted to dpm using a quench curve.

RESULTS

The measured doubling time for the rat aorta smooth muscle cells in the 10% serum growth medium used was 23.7 ± 1.6 hr and cells usually became confluent at about 4 days. Confluent cultures superfused with [^{14}C]arachidonic acid synthesized primarily prostacyclin (identified as its stable breakdown product 6-keto PGF_{1a}) as the major component together with lesser quantities of prostaglandins E_2, F_{2a}, and D_2 (Fig. 1). Prostacyclin production averaged approximately 90 nmol per mg of cell protein and was confirmed by bioassay of the supernatant medium at varying times following addition of arachidonic acid to the cultures and by gas–liquid chromatography–mass spectrometry of appropriate derivatives as described previously (23).

Release of prostacyclin was directly related to the concentration of arachidonic acid with which the cultures were superfused in the range from 3 μM to 25 μM. The release of prostacyclin was extremely rapid, occurring in a pulse lasting approximately 1–2 min fol-

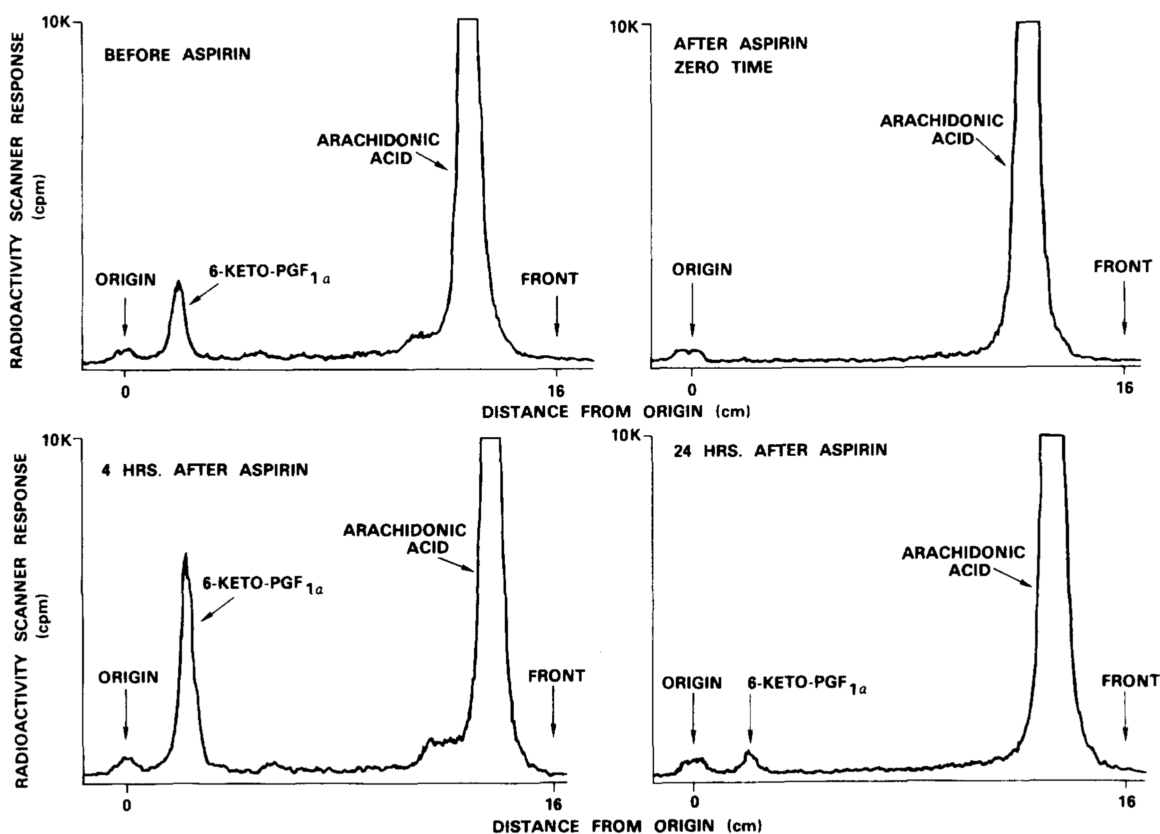

Fig. 1 Recovery of prostacyclin synthetase in cultured rat aorta smooth muscle cells following aspirin treatment. Confluent cultures of rat aorta smooth muscle cells were incubated with HNCTC-135 growth medium containing aspirin (200 μM) at 37°C for 30 min. The medium was replaced with fresh growth medium containing 10% serum. At intervals of 4 and 24 hr the medium was removed, the cells were washed, and the monolayers were superfused with [1-^{14}C]arachidonic acid (4 μg, 0.75 μCi) for 5 min. The products were extracted from the medium and analyzed by TLC as described in Methods. The developed TLC plates were scanned in a Vanguard Scanner at an attenuation of 10 K. The prostaglandin products PGE_2, PGD_2, and PGF_2 were visualized by radioautography and quantitated by scraping the plates and counting as described in Methods.

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lowing exposure to arachidonic acid. That the reaction was not substrate-limited can be readily ascertained from the profile shown in Fig. 1, which indicates that typically the reaction became self-limiting when 50–75% of the arachidonic acid substrate still remained in the medium.

Following treatment with aspirin (0.2 mM) for 30 min, the ability to synthesize prostacyclin and other prostaglandins was completely destroyed (Fig. 1, **Table 1**). When the aspirin-containing medium was removed and replaced with fresh medium, the prostacyclin-synthesizing ability of the cells recovered rapidly, reaching a level within 4 hr which was approximately twice that in the cells immediately before aspirin treatment (Fig. 1, panel 3). In cultures that were followed for longer periods, prostacyclin synthesis then declined to about 15% of control levels at 24 hr (Fig. 1, panel 4). Recovery of the minor products PGE_2, PGD_2, and PGF_{2a} paralleled that of prostacyclin (**Table 2**) confirming that the cyclooxygenase (rather than the prostacyclin endoperoxide isomerase) was the aspirin-labile component being measured.

In order to determine the mechanism underlying this recovery, aspirin-inactivated cultures were incubated with fresh medium in the presence and absence of cycloheximide (20 μg/ml) or actinomycin D (2 μg/ml) as inhibitors of protein and RNA synthesis, respectively. Cycloheximide completely blocked the recovery (**Fig. 2**, lower curve) whereas actinomycin D allowed recovery to occur at about 50% of the control rate (Fig. 2, upper curve). The maximum recovered levels of the enzyme were essentially the same in both control and actinomycin-treated cultures. However, actinomycin D increased the time taken to reach these maximum levels and also prevented the subsequent decline of enzyme levels that occurred in control cultures.

It was found that the recovery of the enzyme following aspirin treatment was completely dependent upon a factor present in the calf serum component of the fresh growth medium. Cells incubated either in NCTC 109 medium alone ('plain' media) or in serum medium that was removed from confluent cell cultures ('spent' medium) did not support recovery of the enzyme (**Fig. 3**). The recovery factor in serum was inactivated by heating at 80°C. The factor was not related to platelet-derived growth factor since equal activities were found in serum prepared from whole blood and both platelet-rich and platelet-poor plasma.

TABLE 1. Recovery of prostacyclin synthesis in aspirin-inactivated rat aorta smooth muscle cells: stimulating activity of whole serum, lipoprotein-free serum, and isolated lipoproteins

Treatment	Picomoles PGI_2 Synthesized per Culture per 5 min
Before aspirin (ASA)	210
After aspirin	2
3 Hours after aspirin	28
10% HDL	35
10% VLDL + LDL	14
10% Lipoprotein-free infranatant	69
10% Serum	196

Confluent cultures of rat aorta smooth muscle cells (10 cm^2) were washed with HNCTC-135 medium and incubated for 30 min in the same medium containing aspirin (ASA, 0.2 mM). The treated cultures were washed free from aspirin and then incubated at 37°C in HNCTC-135 medium alone (plain medium) or in HNCTC-135 supplemented with the indicated lipoprotein subfractions at a level equivalent to their concentrations in 10% serum. After 3 hr the medium was removed and the washed cells were superfused with HNCTC-135 medium (0.5 ml) containing [1-^{14}C]arachidonic acid (2 μg, 55 Ci/M) for 5 min. Additional cultures were submitted to similar incubations either before or immediately after aspirin treatment. The media were extacted and 6-keto $PGF_{1\alpha}$ was separated by TLC in solvent I_w as described in Methods. Values given are the means of duplicate cultures. Note that none of the individual lipoprotein fractions stimulated recovery above the level achieved in plain medium alone. The incomplete recovery of activity in the lipoprotein-free infranatant may be due to the presence of residual KBr in this fraction.

TABLE 2. Restoration of prostaglandin synthesis in aspirin-inactivated rat smooth muscle cells by serum factor

	Picomoles Synthesized per Culture per 5 min			
		0 Time	3 hr after ASA	
Prostaglandin	No Treatment	After ASA	Plain Medium	10% Serum
6-Keto $PGF_{1\alpha}$	114.2 ± 3.9	2.1 ± 0.6	3.4 ± 0.7	133 ± 7.2
PGE_2	14.4 ± 0.6	0.2 ± 0.1	0.3 ± 0.2	16.2 ± 3.1
PGD_2	7.7 ± 0.0	0.3 ± 0.2	0.1 ± 0.3	9.0 ± 0.8
$PGF_{2\alpha}$	6.9 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	9.1 ± 1.7

Confluent cultures (10 cm^2) of rat aorta smooth muscle cells that had been explanted in 10% serum medium 4 days earlier, were incubated for 30 min with ASA (0.2 mM) in HNCTC-135 medium alone (plain medium) or with the same medium supplemented with 10% fetal bovine serum. One-half of the treated cultures together with additional untreated cultures were incubated for a further 3 hr at 37°C. Medium was removed and cultures were superfused with the same medium containing [1-^{14}C]arachidonic acid (0.375 μCi; 55 Ci/M) for 5 min. Note that treatment with fresh serum restored production of all prostaglandin classes to approximately the same extent, indicating that the serum factor acts to increase synthesis of the cyclooxygenase.

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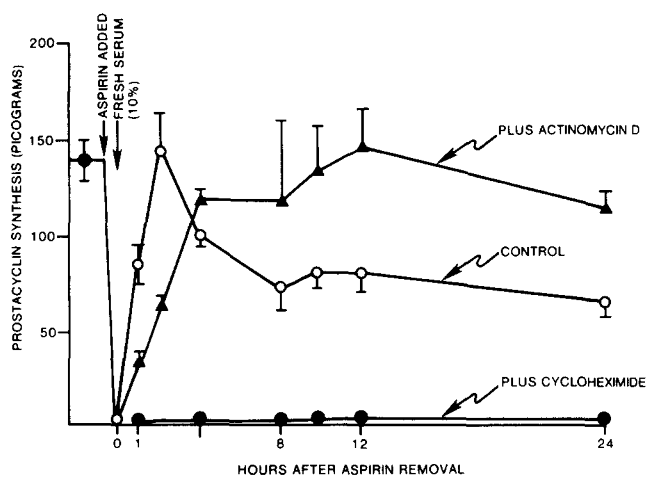

Fig. 2 Recovery of prostacyclin synthesis in aspirin-treated rat aorta smooth muscle cells: inhibition by cycloheximide. Confluent cultures were treated with aspirin for 30 min as described in the legend to Fig. 1. The washed cells were incubated with fresh growth medium for varying intervals and recovery of prostacyclin synthesis was tested. Medium contained 10% serum in HNCTC-135 alone (control), or supplemented with cycloheximide (20 μg/ml) or actinomycin D (2 μg/ml), sufficient to produce >90% inhibition of RNA synthesis.

Several experiments were carried out to determine whether the cyclooxygenase recovery-factor activity in serum was associated with a low molecular weight substance bound to the serum proteins. It was found that the activity was recovered substantially in the upper layer following filtration of serum through a Diaflo filter with a molecular weight cut-off of approximately 30,000 (**Fig. 4**). In addition, serum samples were delipidized by treatment with ethanol at

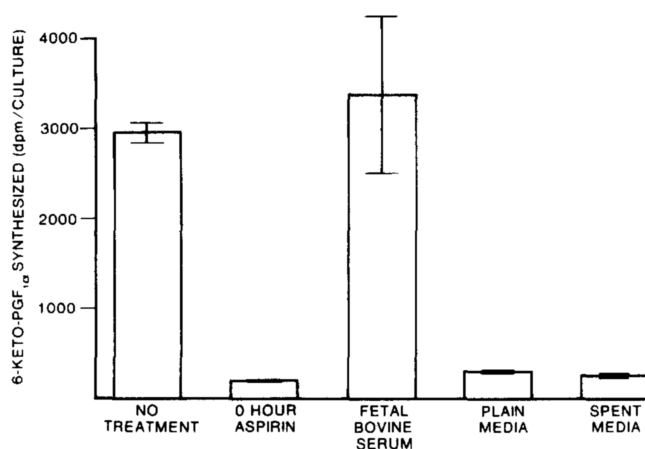

Fig. 3 Recovery of prostacyclin synthesis after aspirin treatment: requirement for a serum factor. Monolayers of rat aorta smooth muscle cells were treated with aspirin for 30 min. The aspirin-containing medium was removed and replaced with the additions as indicated. Medium was HNCTC-135 alone (plain media) or supplemented with 10% fetal bovine serum or spent media. Spent medium consisted of 10% fetal bovine serum in NCTC-135 that was removed from confluent cultures following 4 days of growth. Recovery of prostacyclin synthesis was measured after 3 hr as described in Methods using [1-^{14}C]arachidonic acid as substrate.

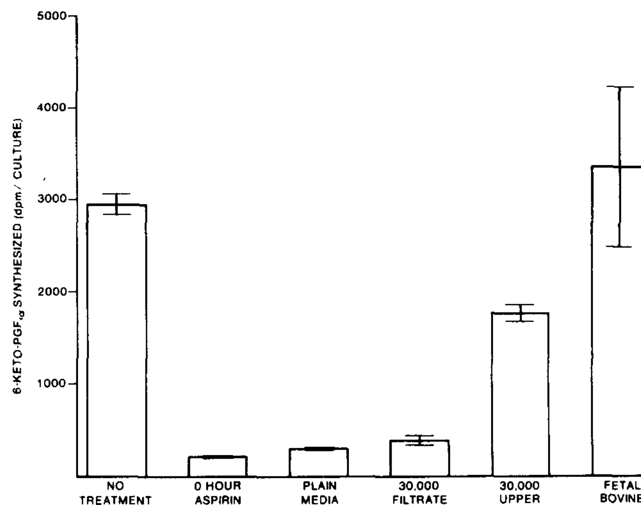

Fig. 4 Recovery of prostacyclin synthesis after aspirin treatment: molecular size of the serum factor. Bovine serum was filtered through Amicon filters having a molecular weight cut-off of approximately 30,000. The filtrate and impermeate were tested at a level of 10% in the growth medium for ability to support recovery of aspirin-inactivated cells. The bulk of the activity present in serum was present in the upper impermeate fraction, and the filtrate was without activity.

−70°C as described previously (22). Both the lipid-extractable and the reconstituted delipidized serum protein fractions were tested for recovery activity on aspirin-inactivated cells. Although this treatment resulted in about 50% inactivation of the recovery factor, the residual activity was found to be associated with the protein and not the lipid fractions. In further experiments, serum was separated by ultracentrifugation into the VLDL + LDL and HDL subfractions and each was tested individually. Essentially, no activity was found in any of the lipoproteins and the activity that was recovered remained in the lipoprotein-free supernatant (Table 1).

When fresh culture medium containing 10% serum was added to confluent 4-day cultures of aspirin-untreated cells, basal levels of prostacyclin synthesis were increased about 2.5-fold during a 3–8 hr period (**Fig. 5**). Not only was this increase completely blocked by simultaneous addition of cycloheximide, but furthermore, in cycloheximide-treated cells, basal levels of prostacyclin synthesis declined almost to zero within 2 hr of addition of cycloheximide. This was followed by a slow recovery of the enzyme to about 50% of normal levels by 12 hr. Following addition of fresh serum-containing medium, synthesis of all prostaglandin classes was increased proportionately (Table 2) indicating that the effects observed were directly on the cyclooxygenase enzyme. To determine whether the activity was peculiar to fetal bovine serum, serum samples from other species including adult human and guinea pig were also tested and gave similar enhancement of prostacyclin synthesis (**Table 3**).

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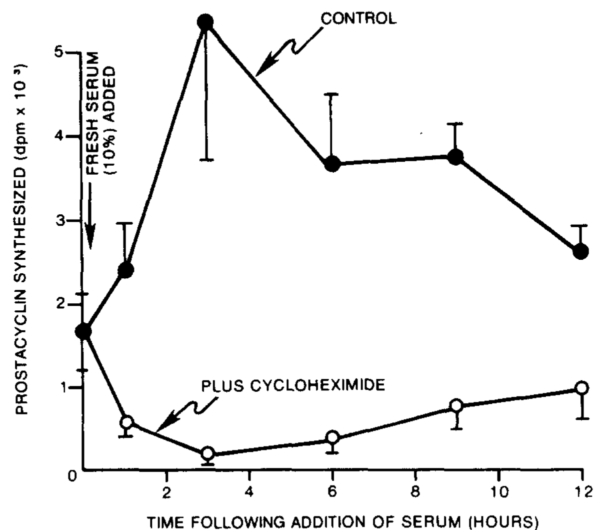

Fig. 5 Stimulation of prostacyclin synthesis by fresh serum and inhibition by cycloheximide. Growth medium was removed from confluent cultures of rat aorta smooth muscle cells after 4 days of growth and replaced with fresh medium containing 10% fetal bovine serum. To one set of cultures, cycloheximide (20 μg/ml) was added. The growth medium was removed from selected cultures at the indicated intervals and prostacyclin synthesis from [^{14}C]arachidonic acid was measured as described in Methods.

To investigate whether the serum factor was also required by other cells, prostaglandin synthesis was measured in a number of other cell types both before and after aspirin treatment. As indicated in **Table 4**, prostaglandin E_2 was the major product synthesized by all the other cell types examined. In all cases PGE_2 synthesis was essentially completely inactivated by treatment with 0.2 mM aspirin for 30 min (Table 4, column 3), and for all five cell lines tested, recovery of the cyclooxygenase required the presence of serum factor (Table 4, columns 4 and 5).

A number of serum constituents were tested for their ability to replace the activity of 10% whole serum, including α-tocopherol (1 to 10 μg/ml), insulin (10 μg/ml), estrogen (1 μg/ml), HDL (50 μg/ml), platelet-derived growth factor and fibroblast growth factor (FGF) (2 μg/ml). None gave significant stimulation of recovery over the basal serum-free NCTC-135 medium

TABLE 3. Species distribution of serum stimulation factor

Serum Additions	Prostacyclin Synthesized
	dpm per Culture per 5 min
None (control)	9500 ± 1200
Serum free	9400 ± 2450
10% Fetal bovine	33500 ± 2250
10% Human serum	31500 ± 300
10% Newborn calf	33000 ± 1800
10% Guinea pig serum	29500 ± 650

Confluent cultures of RSM that had been explanted in 10% fetal bovine serum medium 4 days earlier were washed once and treated with fresh HNCTC-135 medium with the indicated serum additions for 3 hr. Cells were then washed two times with saline and superfused with 2.5 ml of HNCTC medium plus [^{14}C]arachidonic acid (0.375 μCi, 55 Ci/M) for 5 min. The medium was extracted and analyzed for PGI_2 synthesized as described in Methods. Similar results were obtained in aspirin-treated cells using serum samples from eight individual human donors and ten samples of fetal bovine and newborn calf serum. Serum from the other species listed was not tested against aspirin-inactivated cells.

alone. However, addition of purified epidermal growth factor (EGF), over a concentration range of 4 ng to 40 μg per ml, progressively restored prostacyclin synthesis after aspirin inactivation, with maximum stimulation occurring at approximately 40 ng/ml (**Fig. 6**).

DISCUSSION

Since the measured doubling time of the cells in 10% serum growth medium is 24–30 hr, the rapid recovery of cyclooxygenase enzyme from zero to normal levels within 2 hr following addition of serum must represent a relatively selective activation of synthesis of the cyclooxygenase protein over that of the average cell protein. Since the recovery is blocked by cycloheximide but not by actinomycin D, it may be concluded that utilization of pre-existing mRNA is probably involved. It was observed in several experiments that the recovery of enzyme following aspirin inactivation was essentially 100% of the baseline level (i.e., before aspirin treatment level) by about 1 hr following addition of serum, increasing to a maximum at 2–4 hr which was frequently

TABLE 4. Total prostaglandin synthesis

Cell Line	Major Product	Before Aspirin	After Aspirin: 0 Time	After Aspirin: 3-Hr Incubation with NCTC Alone	After Aspirin: 3-Hr Incubation With NCTC & Serum
		pmol per culture per 5 min			
RSM-1	PGI_2	56.6 ± 2.3	1.9 ± 0.2	3.8 ± 0.2	67.3 ± 17.3
K-Balb 3T3	PGE_2	28.5 ± 8.6	2.1 ± 0.5	10.7 ± 1.2	49.3 ± 0.2
SV-40 3T3	PGE_2	41.7 ± 5.2	2.9 ± 0.1	9.2 ± 0.1	36.6 ± 4.6
REF-9	PGE_2	11.8 ± 1.1	0.3	2.5 ± 1.9	14.3 ± 4.5
NRK	PGE_2	33.5 ± 6.7	3.8 ± 1.7	2.9 ± 0.6	24.7 ± 1.1

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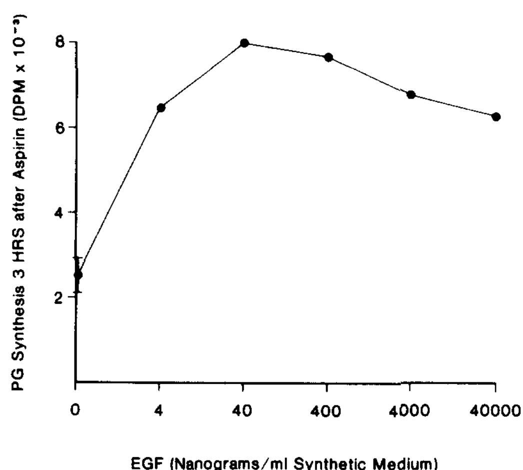

Fig. 6 Stimulation, by epidermal growth factor, of cyclooxygenase recovery after aspirin treatment. Confluent monolayers of rat aorta smooth muscle cells were treated with aspirin (200 μM) for 30 min. The cells were washed and the growth medium was replaced with HNCTC-135 medium supplemented with the indicated concentrations of purified mouse epidermal growth factor (concentration from 1 ng to 10 μg per 0.25 ml culture). After 3 hr the medium was removed and prostacyclin synthesis from [1-^{14}C]arachidonic acid was measured as described in Methods. Results are mean of triplicate cultures for each EGF concentration.

2 or 3 times the basal level, and then falling to levels which were only 15% to 50% of the baseline by 24 hr. The reason for the overshoot of basal levels is probably related to the fact that cultures in 4-day-old, i.e., 'spent' medium, are probably exposed to suboptimal levels of serum factor. This is confirmed in those studies in which the cyclooxygenase levels in non-aspirin-treated cells increase rapidly after addition of serum (Fig. 4 and Tables 2 and 3). The reason for the subsequent decline by 24 hr and the prevention of this decline by the presence of actinomycin D is not understood but may well be related to the mechanisms that operated in cells to regulate the endogenous levels of the cyclooxygenase enzyme.

Studies with molecular filters indicated that the molecular weight of the serum factors was greater than 30,000. However, the activity of serum could not be duplicated by a number of macromolecular constituents of serum having greater molecular weights than this nor by lipid-soluble constituents bound to these proteins. Although the molecular weight of epidermal growth factor from mouse submaxillary gland is only about 6000, it is bound in serum to a carrier protein which has a molecular weight of approximately 30,000 (24).

Quantities of EGF ranging from 1 ng to 20 μg per ml progressively stimulated recovery of prostacyclin synthesis after aspirin. Over a broad range of concentrations, it partially replaced the stimulatory activity of 10% fresh serum (Fig. 6). In other experiments EGF concentrations of 20 ng/ml replaced about 60% of the activity of fresh 10% serum (**Table 5**). That additional components are probably present in serum is suggested by the fact that when added to 'spent' medium, the same concentration of EGF fully replaced the activity of fresh serum.

In addition to the possible role that serum EGF levels may play in recovery of the vasculature following aspirin treatment, this finding may be of more general significance. Cyclooxygenase is a prime example of an enzyme with activity which is regulated by self-inactivation (23). Thus the hydroperoxy fatty acid intermediates formed during the action of the enzyme react covalently with the enzyme protein resulting in its permanent inactivation. This phenomenon also operates under physiological conditions in intact cells using releasing agents such as thrombin (17). Reactivation of the prostaglandin synthetase system after a pulse of activation will therefore require synthesis of new enzyme. The rapid recovery in as little as 20–30 min

TABLE 5. Comparative restoration of prostacyclin synthetase levels in aspirin-inactivated cells by EGF, fresh serum, spent medium, and EGF-supplemented spent medium

Treatment	Picograms PGI_2 Synthesized per Culture per 5 min
None	197 ± 24
Aspirin–plain medium, 3 hr	27 ± 12
Aspirin–fresh serum, 3 hr	253 ± 20
Aspirin–spent medium, 3 hr	46 ± 7
Aspirin–plain medium + 10 ng EGF, 3 hr	80 ± 3
Aspirin–plain medium + 20 ng EGF, 3 hr	160 ± 30
Aspirin–spent medium + 10 ng EGF, 3 hr	154 ± 24
Aspirin–spent medium + 20 ng EGF, 3 hr	296 ± 9

Confluent 3-day cultures of rat smooth muscle cells were treated with aspirin (200 μM) for 30 min and washed and the medium was replaced with the indicated additions. After 3 hr the cells were superfused with [^{14}C]arachidonic acid and prostacyclin synthesis during the next 5 min was measured. Spent medium is medium removed from the confluent cultures after 3 days growth. Note that 20 ng/ml of EGF replaced about 60% of the activity of fresh serum and that, in the presence of the additional components present in the spent medium, it is fully as active as fresh serum.

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after aspirin removal, together with the requirement for EGF, may therefore represent a recovery mechanism for this type of enzyme following desensitization. Preliminary evidence from self-inactivation experiments following arachidonic acid stimulation of vascular smooth muscle cells supports this interpretation.

Manuscript received 11 June 1984.

REFERENCES

1. Hamberg, M., J. Svensson, and B. Samuelsson. 1975. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. USA.* **72:** 2994–2998.
2. Samuelsson, B., S. Hammarstrom, R. C. Murphy, and P. Borgeat. 1980. Leukotrienes and slow-reacting substance of anaphylaxis (SRS-A). *Allergy.* **35:** 375–381.
3. Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol.* **231:** 232–235.
4. Roth, G. J., N. Stanford, and P. W. Majerus. 1975. Acetylation of prostaglandin synthase by aspirin. *Proc. Natl. Acad. Sci. USA.* **72:** 3073–3076.
5. Svensson, J., M. Hamberg, and B. Samuelsson. 1975. Prostaglandin endoperoxides. IX. Characterization of rabbit aorta contracting substance (RCS) from guinea pig lung and human platelets. *Acta Physiol. Scand.* **94:** 222–228.
6. Bailey, J. M. 1979. Prostacyclins, thromboxanes and cardiovascular disease. *Trends Biochem. Sci.* **4:** 68–71.
7. Ross, R., and J. A. Glomset. 1976. The pathogenesis of atherosclerosis. *N. Engl. J. Med.* **295:** 369–420.
8. Bailey, J. M., and J. Butler. 1973. Anti-inflammatory drugs in experimental atherosclerosis. Part I. Relative potencies for inhibiting plaque formation. *Atherosclerosis.* **17:** 151–153.
9. Aspirin Myocardial Infarction Study Research Group. 1980. A randomized, controlled trial of aspirin in persons recovered from myocardial infarction. *J. Am. Med. Assoc.* **243:** 661–669.
10. The Anturane Reinfarction Trial Research Group. 1980. Sulfinpyrazone in the prevention of sudden death after myocardial infarction. *N. Engl. J. Med.* **302:** 250–256.
11. Moncada, S., A. G. Herman, E. A. Higgs, and J. R. Vane. 1977. Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the antithrombotic properties of vascular endothelium. *Thromb. Res.* **11:** 323–344.
12. Weksler, B. B. 1983. Differential inhibition by aspirin or vascular and platelet prostaglandin synthesis in atherosclerotic patients. *N. Engl. J. Med.* **308:** 800–805.
13. Weksler, B. B., A. J. Marcus, and E. A. Jaffee. 1977. Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine cells. *Proc. Natl. Acad. Sci. USA.* **74:** 3922–3926.
14. Marcus, A. J., B. B. Weksler, and E. A. Jaffee. 1978. Enzymatic conversion of prostaglandin endoperoxide H_2 and arachidonic acid to prostacyclin by cultured human endothelial cells. *J. Biol. Chem.* **253:** 7138–7141.
15. Willems, C., and W. G. Van Aken. 1979. Production of prostacyclin by vascular endothelial cells. *Haemostasis.* **8:** 266–273.
16. Spector, A. A., J. C. Hoak, G. L. Fry, G. M. Dennins, L. L. Stoll, and J. B. Smith. 1980. Effect of fatty acid modification on prostacyclin production by cultured human endothelial cells. *J. Clin. Invest.* **65:** 1003–1012.
17. Salata, K. F., J. D. Whiting, and J. M. Bailey. 1980. Regeneration of prostacyclin synthetase activity in cultured vascular cells following aspirin treatment. *Adv. Prostaglandin Thromboxane Res.* **6:** 537–541.
18. Baenziger, N. L., P. R. Becherer, and P. W. Majerus. 1979. Characterization of prostacyclin synthesis in cultured human arterial smooth muscle cells, venous endothelial cells and skin fibroblasts. *Cell.* **16:** 967–974.
19. Silberbauer, K., H. Sinzinser, and M. Winter. 1978. Prostacyclin production by vascular smooth muscle cells. *Lancet.* **24:** 1356–1357.
20. Gordon, J. L., and J. D. Pearson. 1978. Effects of sulphinpyrazone and aspirin on prostaglandin I_2 (prostacyclin) synthesis by endothelial cells. *Br. J. Pharmacol.* **64:** 481–483.
21. Whiting, J., K. Salata, and J. M. Bailey. 1980. Aspirin: an unexpected side effect on prostaglandin synthesis in cultured vascular smooth muscle cells. *Science.* **210:** 663–665.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
23. Bailey, J. M., R. W. Bryant, and K. Salata. 1983. Characterization of 11-HETE and 15-HETE, together with prostacyclin, as major products of the cyclooxygenase pathway in cultured rat aorta smooth muscle cells. *J. Lipid Res.* **24:** 1419–1428.
24. Taylor, J., W. Mitchell, and S. Cohen. 1974. Characterization of the binding protein for epidermal growth factor. *J. Biol. Chem.* **249:** 2188–2194.

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